Attaching substances to micro-organisms

Buist, Girbe; Leenhouts, Cornelis Johannes ; Venema, Gerard ; Kok, Jan

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/31, 1/20, C07K 14/315, 14/195, 14/37, C12N 9/36, A61K 38/02, A23L 1/03, G01N 33/68, B01J 20/00

A1

(11) International Publication Number: **WO 99/25836**

(43) International Publication Date: 27 May 1999 (27.05.99)

(21) International Application Number: PCT/NL98/00655

(22) International Filing Date: 12 November 1998 (12.11.98)

(30) Priority Data:
97203539.8 13 November 1997 (13.11.97) EP

(71) Applicant *(for all designated States except US)*: RIJKSUNIVERSITEIT GRONINGEN [NL/NL]; Broerstraat 5, NL–9712 CP Groningen (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BUIST, Girbe [NL/NL]; Jaltadaheerd 50, NL–9737 HB Groningen (NL). LEENHOUTS, Cornelis, Johannes [NL/NL]; Tussenkoelen 6, NL–9753 KX Haren (NL). VENEMA, Gerard [NL/NL]; Kerklaan 26, NL–9751 NN Haren (NL). KOK, Jan [NL/NL]; Van Hamelstraat 34, NL–9714 HL Groningen (NL).

(74) Agent: OTTEVANGERS, S., U.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL–2587 BN The Hague (NL).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ATTACHING SUBSTANCES TO MICRO–ORGANISMS

(57) Abstract

The invention relates to surface display of proteins on micro–organisms via the targeting and anchoring of heterologous proteins to the outer surface of cells such as yeast, fungi, mammalian and plant cells, and bacteria. The invention provides a proteinaceous substance comprising a reactive group and at least one attaching peptide which comprises a stretch of amino acids having a sequence corresponding to at least a part of the consensus amino acid sequence listed in figure 10 and comprises a method for attaching a proteinaceous substance to the cell wall of a micro–organism comprising the use of said attaching peptide.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Title: Attaching substances to micro-organisms

Heterologous surface display of proteins (Stahl and Uhlen, TIBTECH May 1997, 15, 185-192) on recombinant micro-organisms via the targeting and anchoring of heterologous proteins to the outer surface of host-cells such as yeast, fungi, mammalian and plant cells, and bacteria has been possible for several years. Display of heterologous proteins at the surface of these cells has taken many forms, varying from the expression of reactive groups such as antigenic determinants, heterologous enzymes, (single-chain) antibodies, polyhistidyl tags, peptides, and other compounds. Heterologous surface display has been applied as a tool for applied and fundamental research in microbiology, molecular biology, vaccinology and biotechnology, and several patent applications have been filed.

Yet another application of bacterial surface display has been the development of live-bacterial-vaccine delivery systems. The cell-surface display of heterologous antigenic determinants has been considered advantageous for the induction of antigen-specific immune responses when using live recombinant cells for immunisation. Another application has been the use of bacterial surface display in generating whole-cell bioadsorbents or biofilters for environmental purposes, microbiocatalysts, and diagnostic tools.

In general, one has used chimeric proteins consisting of an anchoring or targeting part specific and selective for the recombinant organism used and has combined this part with a part comprising a reactive group as described above. A well known anchoring part for example comprise the so-called LPXTG box, that binds covalently to a *Staphylococcus* bacterial surface, i.e. in the form of a fully integrated membrane protein. In this way, chimeric proteins are composed of at least two (poly)peptides of different genetic origin joined by a normal peptide bond. For example, in patent application WO

94/18830 relating to the isolation of compounds from complex mixtures and the preparation of immobilised ligands (bioadsorbents), a method has been claimed for obtaining such a ligand which comprises anchoring a binding protein in or at the exterior of the cell wall of a recombinant cell. Said binding protein is essentially a chimeric protein produced by said recombinant cell, and is composed of an N-terminal part, derived from for example an antibody, that is capable of binding to a specific compound joined with a C-terminal anchoring part, derived from an anchoring protein purposely selected for being functional in the specific cell chosen. In patent application WO 97/08553 a method has been claimed for the targeting of proteins selectively to the cell wall of *Staphylococcus spp*, using as anchoring proteins long stretches of at least 80-90 amino acid long amino acid cell wall-targeting signals derived from the lysostaphin gene or amidase gene of *Staphylococcus* which encode for proteins that selectively bind to *Staphylococcus* cell wall components.

Vaccine delivery or immunisation via attenuated bacterial vector strains expressing distinct antigenic determinants against a wide variety of diseases is now commonly being developed. Recently, mucosal (for example nasal or oral) vaccination using such vectors has received a great deal of attention. For example, both systemic and mucosal antibody responses against an antigenic determinant of the hornet venom were detected in mice orally colonised with a genetically engineered human oral commensal *Streptococcus gordonii* expressing said antigenic determinant on its surface (Medaglini et al., PNAS 1995, 2; 6868-6872). Also, a protective immune response could be elicited by oral delivery of a recombinant bacterial vaccine wherein tetanus toxin fragment C was expressed constitutively in *Lactococcus lactis* (Robinson et al., Nature Biotechnology 1997, 15; 653-657). Especially mucosal immunisation as a means of inducing IgG and secretory IgA antibodies directed against specific pathogens

of mucosal surfaces is considered an effective route of vaccination. Immunogens expressed by bacterial vectors are presented in particulate form to the antigen-presenting cells (for example M-cells) of the immune system and should therefore be less likely to induce tolerance than soluble antigens. In addition, the existence of a common mucosal immune system permits immunisation on one specific mucosal surface to induce secretion of antigen-specific IgA, and other specific immune responses at distant mucosal sites. A drawback to this approach is the potential of the bacterial strain to cause inflammation and disease in itself, potentially leading to fever and bacteraemia. An alternative approach avoids the use of attenuated bacterial strains that may become pathogenic themselves by choosing recombinant commensal bacteria as vaccine carriers, such as *Streptococcus spp.* and *Lactococcus spp.*

However, a potential problem with such recombinant organisms is that they may colonise the mucosal surfaces, thereby generating a long term exposure to the target antigens expressed and released by these recombinant micro-organisms. Such long term exposure can cause immune tolerance. In addition, the mere fact alone that such organisms are genetically modified and contain recombinant nucleic acid is meeting considerable opposition from the (lay) public as a whole, stemming from a low level of general acceptance for products containing recombinant DNA or RNA. Similar objections exist against the use of (even attenuated) strains of a pathogenic nature or against proteins or parts of proteins derived from pathogenic strains. However, as explained above, present techniques of heterologous surface display of proteins in general entail the use of anchoring or targeting proteins that are specific and selective for a limited set of micro-organisms which in general are of recombinant or pathogenic nature, thereby greatly restricting their potential applications.

The invention provides substances and methods to anchor or attach said substances to a cell-wall or cell-wall component of a wide range of micro-organisms. A preferred embodiment of the invention provides substances and methods to attach said substances to non-recombinant micro-organisms. Said substances provided by the invention are not limited to (chimeric) proteins alone, but can be fully or only partly of a peptide nature, whereby a peptide part is (covalently) joined to a non-peptide moiety. The invention provides a proteinaceous substance comprising at least one stretch of amino acids derived from a first micro-organism which substance is capable of attaching to a cell-wall of a second micro-organism. Said substance according to the invention is for example produced by a first micro-organism (for example a micro-organism from which the knowledge about the sequence of said stretch of amino acids originates, but another (recombinant) micro-organism can produce said substance as well). After its production said substance is harvested, optionally stored for future use, and then brought in contact with said second micro-organism, where it attaches to its cell-wall. Alternatively, said substance is produced synthetically, by using established peptide synthesis technology. A preferred embodiment of the invention provides a substance wherein said second micro-organism is a non-recombinant micro-organism. With a substance provided by the invention it is now possible to attach or anchor for a example a heterologous or chimeric protein produced by a recombinant micro-organism to an innocuous non-recombinant micro-organism.

A preferred embodiment of the invention provides a proteinaceous substance wherein said stretch of amino acids has a sequence corresponding to a consensus sequence listed in figure 10, or wherein said stretch of amino acids (herein also called attaching peptide) has a sequence corresponding to a sequence selected from those listed in figure 11, or a homologous sequence derived from another species. The

sequences listed in figure 11, and sequences homologuous thereto, are found in a variety of species, both micro-organisms and higher organisms, an example of such a higher organism is *C. elegans*. Preferably, the attaching peptide is derived from any one of the proteins listed in figure 11, more preferably said attaching peptide comprises an amino acid sequence as shown in figure 10, or a sequence derived thereof. For example, the invention provides a proteinaceous substance wherein said attaching peptide is derived from the major peptidoglycan hydrolase of *Lactococcus lactis*.

Yet another preferred embodiment of the invention provides a proteinaceous substance wherein said second micro-organism is selected from any of the group of Gram-positive bacteria and Gram-negative bacteria. Examples are micro-organisms, such as *Bacillus subtilis, Clostridium beijerinckii, Lactobacillus plantarum, Lb. buchneri, Listeria inocua, Streptococcus thermophilus, Enterococcus faecalis, E. coli,* and others.

The invention provides a proteinaceous substance which is additionally comprising a reactive group. For example, the invention provides a proteinaceous substance comprising a reactive group such as an antigenic determinant, heterologous enzyme, (single-chain) antibody or fragment thereof, polyhistidyl tag, fluorescing protein, luciferase, binding protein or peptide, or another substance such as an antibiotic, hormone, non-peptide antigenic determinant, carbohydrate, fatty acid, aromatic substance and reporter molecule, and an anchoring or targeting protein or part thereof (herein also called attaching peptide) useful in heterologous surface display which is both broadly reactive with cell wall components of a broad range of micro-organisms.

For example, the invention provides a substance wherein said reactive group is a non-protein moiety, for example is selected from the group of antibiotics, hormones, aromatic substances and reporter molecules. Said substance is

constructed by binding for example an antibiotic, such as penicillin or tetracycline, but various other antibiotics can be used, or a hormone, such as a steroid hormone, or any other compound to an attaching peptide provided by the invention. Such binding can be achieved by various techniques known in the art, and thereby can label or "flag" the attaching peptide. A preferred example is the binding of an attaching peptide to a reporter molecule such as FITC, or HRPO, whereby tools are generated that can be used in diagnostic assay whereby micro-organisms having peptidoglycan are detected. Similarly, an attaching peptide with an antibiotic bound thereto can be used *in vivo* by for example parenteral administration into the bloodstream of humans or animals or *in vitro* to bind to such micro-organisms having peptidoglycan, thereby increasing the concentration of antibiotic around said organism, which then gets killed by the antibiotic action.

The invention provides a substance wherein said reactive group is a protein moiety, for example selected from the group of antigenic determinants, enzymes, (single-chain) antibodies or fragments thereof, polyhistidyl tags, fluorescing proteins, binding proteins or peptides. For example, the invention provides a protein, which comprises as reactive group a protein or (poly)peptide. Also, the invention provides a nucleic acid molecule encoding a protein provided by the invention. Such a nucleic acid molecule (being single- or double stranded DNA, RNA or DNA/RNA) at least comprises nucleic acid sequences specifically encoding a attaching peptide as well as nucleic acid sequences specifically encoding the reactive group polypeptide, but can additionally also comprise other nucleic acid sequences, which for example encode a signal peptide, or comprise for example promoter and/or regulatory nucleic acid sequences. The invention also provides a vector comprising a nucleic acid molecule encoding a protein provided by the invention.

The invention provides a proteinaceous substance comprising a reactive group joined with or bound to at least one attaching peptide which comprises a stretch of amino acids corresponding to the consensus amino acid sequence listed in figure 10, said substance capable of attaching or anchoring or binding to a cell wall component of a micro-organism.

Corresponding to is defined as having an amino acid sequence homologous to the consensus amino acid sequence listed in figure 10, or having an amino acid sequence derived of the sequence listed in figure 10 which derived sequence comprises a functionally equivalent stretch of amino acids.

Preferably, the attaching peptide is derived from any one of the proteins listed in figure 11, or a protein having a repeat sequence related or homologous to the sequence listed in figure 10, more preferably said attaching peptide comprises an amino acid sequence as shown in figure 10, or a sequence derived thereof. Homology between the various amino acid sequences of related attaching peptides provided by the invention can for instance be determined by performing a homology search between amino acid sequences, such as for example can be found in a protein database, such as the SWISSPROT, PIR and Genbank databases, using a computer programme, such as the BLAST programme, that can determine homology between amino acid sequences. For example, the invention provides a proteinaceous substance wherein said attaching peptide is derived from the major peptidoglycan hydrolase of *Lactococcus lactis*. The invention provides a proteinaceous substance comprising a reactive compound wherein at least two stretches of amino acids, corresponding to an attaching peptide sequence, are located adjacent to each other, possibly separated by one or more amino acid residues. Said stretches or repeats can be separated by a short distance, for example 3-6 to 10-15 amino acids apart , or by a medium distance 15-100 amino acids apart, or by longer distances (>100 amino acid residues apart). Examples of such

distances can be found in figure 11, but longer distances are also possible. The distances between said stretches or repeats can also be used for an (additional) reactive group, whereby a reactive group is inserted between repeats, thereby allowing an even better anchoring to a cell wall component. A preferred embodiment provided by the invention is a proteinaceous substance comprising a reactive group and at least one attaching peptide which comprises a stretch of amino acids having a sequence corresponding to the consensus amino acid sequence listed in figure 10, wherein said substance is capable of attaching to a cell wall component of a micro-organism, such as can be found among from any of the group of yeast, moulds, Gram-positive bacteria and Gram-negative bacteria. Examples are micro-organisms, such as *Bacillus subtilis, Clostridium beijerinckii, Lactobacillus plantarum, Lb. buchneri, Listeria inocua, Streptococcus thermophilus, Enterococcus faecalis, E. coli,* and others. A preferred embodiment provided by the invention is a proteinaceous substance which is capable of attaching to a cell wall component of a conventional (non-recombinant) micro-organism. In this embodiment, the invention provides for example non-recombinant organisms which displaying heterologous proteins, these may colonise the mucosal surfaces without causing problems such as immune tolerance, since they do not generate a long term exposure to the target antigens expressed. In addition, the mere fact alone that such organisms provided by the invention are not genetically modified and do not contain recombinant nucleic acid will alleviate the opposition from the (lay) public as a whole against recombinant micro-organisms, which is stemming from a low level of general acceptance for products containing recombinant DNA or RNA. Similar objections that exist against the use of (even attenuated) strains of a pathogenic nature or against proteins or parts of proteins derived from pathogenic strains are now also overcome by the invention, in that is now possible to

attach a proteinaceous substance to a non-recombinant, non-pathogenic micro-organism, such as *L. lactis* which is generally considered as safe. The invention provides a proteinaceous substance comprising a reactive group such as an antigenic determinant, (heterologous) enzyme, (single-chain) antibody or fragment thereof, polyhistidyl tag, fluorescing protein, luciferase, binding protein or peptide, or another compound such as an antibiotic, hormone, non-peptide antigenic determinant, carbohydrate, fatty acid, aromatic compound and reporter molecule, and an anchoring or targeting protein or part thereof (herein also called attaching peptide) useful in heterologous surface display which is both broadly reactive with cell wall components of a broad range of micro-organisms. Said attaching peptide is preferably derived from a micro-organism which is generally recognised as safe (G.R.A.S.), thereby greatly enhancing the potential of applications of the heterologous surface display technique. *Lactococcus lactis* is a non-pathogenic, non-invasive, and non-colonising Gram-positive bacterium which is not adapted for growth in body or even the gut; it does not belong to the commensal species of lactic acid bacteria. *L. lactis* has a history of safe use of several thousand years. The major cell wall hydrolase AcmA of the Gram-positive bacterium *Lactococcus lactis* subsp. *cremoris* MG1363 is an N-acetylmuramidase which is required for cell separation and is responsible for cell lysis during stationary phase. The protein consists of three separate domains (Fig. 9, Buist et al., J. Bacteriol. (1995) 177:1554-1563) of which the first 57 amino acids of the N-terminal domain encompasses the signal peptide needed for secretion. This domain is followed by the active site domain running from the Ala at position 58 to Ser-218. The active site domain was overproduced in and purified from *Escherichia coli* as a thioredoxin fusion protein. The AcmA part was released by proteolytic cleavage with enterokinase and shown to be active *in vitro*. Three homologous repeated regions (or stretches of amino acids) of

35-55, more often 40-50 amino acid residues are present in the C-terminus of for example AcmA which are separated by non-homologous sequences (Fig.10). The repeat sequences of AcmA (cA) can be deleted and additional repeat sequences could be added without impairing cell wall hydrolysing activity *in vitro*. The AcmA deletion derivatives lacking one or two repeat sequences and the protein containing at least one additional repeat were able to bind to lactococcal cells when added from the outside. The derivative lacking all three repeats did not bind to the cells nor did the purified active site domain. The invention provides an attaching peptide that comprise at least one repeat sequence as shown in figure 10 or a sequence that is similar to the sequence of figure 10, similar being defined as comprising at least a part of a consensus sequence as shown in figure 11. Also, attaching peptides are provided by the invention which are comprising amino acid sequences that are derived from a sequence as shown in figure 11. Derived herein meaning among others by comparison with heterologous sequences whereby a consensus sequence is obtained, or derived via conventional amino acid substitutions whereby amino acids are being substituted by like amino acids, or derived via substitutions whereby functional amino acids are being replaced by functionally alike or better amino acids identified by methods such as PEPSCAN techniques or replacement mapping. The invention provides a proteinaceous substance comprising a reactive group and at least one attaching peptide which comprises a stretch of amino acids having a sequence corresponding to at least a part of the consensus amino acid sequence provided in figure 10. Repeats similar to those in AcmA were for example shown to be present in various cell wall hydrolases and other (secreted) proteins of Gram-positive and Gram-negative bacteria and other micro-organisms and constitute a general cell wall binding domain in these proteins. An attaching peptide comprising at least one AcmA repeat or an amino acid sequence similar to the AcmA

repeat provided by the invention represents a general and broadly reactive tool to bind or attach reactive groups such as antigenic determinants, enzymes, antibodies, proteins or peptides to cell walls of micro-organisms. Said repeat comprises a peptide composed of a stretch of amino acids having a sequence corresponding to at least a part of the consensus amino acid sequence provided in figure 10. Furthermore, we also demonstrated that an attaching peptide provided by the invention bound or attached to cells of other, e.g. non-recombinant micro-organisms, such as *Bacillus subtilis, Clostridium beijerinckii, Lactobacillus plantarum, Lb. buchneri, Listeria inocua, Streptococcus thermophilus, Enterococcus faecalis, E. coli,* and others. Binding of the attaching peptide and reactive group joined therewith, as provided by the invention is stable at pH values ranging from 2-10. Moreover, the attaching peptide provided by the invention is, when attached to the cell wall, protected against proteolytic degradation. One embodiment of the invention is a protein wherein the attaching peptide is derived from any of the proteins listed in figure 11. An example of such an attaching peptide is provided in the experimental part of this description wherein an attaching peptide having a sequence as shown in figure 10, or a sequence similar thereto, is used. Furthermore, the invention provides a protein, which comprises as reactive group a protein or (poly)peptide. Also, the invention provides a nucleic acid molecule encoding a protein provided by the invention. Such a nucleic acid molecule (being single- or double stranded DNA, RNA or DNA/RNA) at least comprises nucleic acid sequences specifically encoding a attaching peptide as well as nucleic acid sequences specifically encoding the reactive group polypeptide, but can additionally also comprise other nucleic acid sequences, which for example encode a signal peptide, or comprise for example promoter and/or regulatory nucleic acid sequences. The invention also provides a vector comprising a

nucleic acid molecule encoding a protein provided by the invention. Such a vector can for example be a plasmid, phage, or virus, and can now be constructed using a nucleic acid provided by the invention and routine skills of the art. Examples of such a vector can be found in the experimental part of the description, other examples can e.g. be a baculovirus vector, or comparable vector viruses through which a protein provided by the invention can be expressed or produced in (insect)cells. The invention also provides a host cell or expression system comprising a nucleic acid molecule according to the invention or a vector according to the invention. Such a host cell expressing a protein is in it self provided by the invention as a micro-organism to which a protein provided by the invention is attached. Such a host cell or expression system can for example be a Gram-positive- or Gram-negative bacterium, or a yeast cell or insect cell or plant- or mammalian cell, or even a cell-free expression system such as a reticulocyte lysate, and can now be constructed or obtained using a nucleic acid or vector provided by the invention and routine skills of the art. Examples of such a host cell or expression system can be found in the experimental part of the description, other examples can be obtained using a nucleic acid or vector provided by the invention and routine skills of the art.

The invention provides a method for attaching a substance to the cell wall of a micro-organism comprising the use of an attaching peptide which comprises a stretch of amino acids having a sequence corresponding to at least a part of the consensus amino acid sequence provided in figure 10. An example of the method provided by the invention is anchoring of recombinant poly(peptides), being (chimeric) proteins fused to the cell wall anchoring repeats of AcmA of *Lactococcus lactis* MG1363, to the cell wall of (Gram-positive) bacteria. The recombinant proteins are obtained by the expression of DNA sequences encoding these recombinant (poly)peptides in a

suitable production strain (e.g. *E. coli* or *L. lactis*) and subsequent purification of the expression products. The recombinant proteins are than mixed, either *in vitro* or *in vivo*, with a non-recombinant target bacterium to obtain binding to the cell wall. Another example of the method provided by the invention is anchoring of recombinant poly(peptides), being (chimeric) proteins fused to the cell wall anchoring repeats of AcmA of *Lactococcus lactis*, to the cell wall of said recombinant *Lactococcus lactis* which produces the protein itself. In a preferred embodiment of the method provided by the invention the binding of (purified) proteins to bacterial cells upon addition from the outside, the method is an excellent tool to anchor recombinant proteins or other substances to non-recombinant bacterial cells.

A preferred method according to the invention comprises the use of an attaching peptide which is derived from the major peptidoglycan hydrolase of *Lactococcus lactis*. Another method according to the invention is provided wherein said substance is a (poly)peptide or a protein, for example being part of a protein provided by the invention. Post-translational modifications occurring to such a (poly)peptide or protein are inherent to the host cell or expression system used, a post-translationally modified protein as provided by the invention is therefore also provided. However, yet another method according to the invention is provided wherein said compound is selected from the group composed of antibiotics, hormones, antigenic determinants, carbohydrate chains, fatty acids, aromatic compounds and reporter molecules. Said substance is constructed by binding for example an antibiotic, such as penicillin or tetracycline, but various other antibiotics can be used, or a hormone, such as a steroid hormone, or any other compound to an attaching peptide provided by the invention. Such binding can be achieved by various techniques known in the art, and thereby can label or "flag" the attaching peptide. A preferred example is the

binding of an attaching peptide to a reporter molecule such as FITC, or HRPO, whereby tools are generated that can be used in diagnostic assay whereby micro-organisms having peptidoglycan are detected. Similarly, an attaching peptide with an antibiotic bound thereto can be used *in vivo* by for example parenteral administration into the bloodstream of humans or animals or *in vitro* to bind to such micro-organisms having peptidoglycan, thereby increasing the concentration of antibiotic around said organism, which than can get killed by the antibiotic action. Said micro-organism is preferably selected from any of the group of yeast, moulds, Gram-positive bacteria and Gram-negative bacteria. For example, the experimental part of this description describes mixing of ß-lactamase::cA fusion protein with lactococcal cells which resulted in binding to the cells whereas this was not the case when mature ß-lactamase not joined with an attachement protein was added. Also, fusion of ß-lactamase of *E. coli* and α-amylase of *Bacillus licheniformis* to the attaching peptide provided by the invention and subsequent production of these fusion proteins resulted in active, secreted proteins which were located (attached) in *L. lactis* cell walls. Binding of AcmA and the ß-lactamase::cA fusion protein was also demonstrated to isolated lactococcal cell walls and SDS-washed cell walls (the major part of this fraction is peptidoglycan).

Anchoring of recombinant proteins to non-recombinant micro-organisms such as lactococci (or other bacteria) or fungi, is especially attractive if the use of recombinant bacteria is not desired, e.g. in food processes or as pharmaceuticals for medical use such as in vaccines or in anti-bacterial therapy. The invention provides for example vaccine delivery or immunisation via micro-organisms provided by the invention which are labelled with distinct antigenic determinants, which may be directed against a wide variety of diseases. A protective immune response can for example be elicited by oral delivery of a bacterial vaccine provided by

the invention wherein tetanus toxin fragment C is attached via a protein provided by the invention to a non-recombinant *Lactococcus lactis*. Such immunogens expressed by micro-organisms provided by the invention are presented in particulate form to the antigen-presenting cells (for example M-cells) of the immune system and are therefore less likely to induce tolerance than soluble antigens. In addition, the existence of a common mucosal immune system permits immunisation on one specific mucosal surface to induce secretion of antigen-specific IgA, and other specific immune responses at distant mucosal sites. The invention solves the drawback of earlier bacterial vaccines whereby the potential to flourish on mucosal surfaces of the (attenuated or recombinant) bacterial strain used can cause problems such as inflammation and disease in itself, potentially leading to fever and bacteraemia, or to the induction of immune tolerance. Also, the invention avoids the potential risks that are involved when using recombinant DNA containing bacterial vectors for vaccination. In yet another possible vaccine and vaccine use provided by the invention, certain (killed) micro-organisms with adjuvant properties (such as the mycobacteria used in BCG) are labelled or loaded with a protein or substance composed of an antigenic determinant and an attaching peptide. These micro-organisms than function as adjuvant, thereby greatly enhancing the immune response directed against the specific antigenic determinant. Yet another use provided by the invention comprises anchoring proteins from the outside to a micro-organism which provides a means to present proteins or peptides which normally can not be overexpressed (and/or secreted) by said micro-organism. For example, the subunit B of cholera toxin (CTB) can be overproduced in *E. coli* but expression in *L. lactis* has been unsuccessful until now. The adjuvant activity of CTB in experimental recombinant vaccines is well documented and the ability of CTB or part thereof to bind to GM1 ganglioside on

eucaryotic cell surfaces is of interest with respect to the use of *L. lactis* (or other Gram-positives) in vaccines which specifically require targeting to mucosal surfaces. Yet another medical use provided by the invention is the addition of (purified) antigen::cA fusion proteins *in vivo* by parenteral administration into the bloodstream of humans or animals to combat bacterial infections. In this case the antigen::cA fusion protein is used as a "flag" for the immune system. The antigenic determinant of a protein provided by the invention being a subunit of a vaccine regularly used for the immunisation of humans (preferably children) or animals, e.g. a subunit of the Rubella, Pertusis, Poliomyelitis, tetanus or measles vaccine. After delivery in the bloodstream, the "flag" will bind through the AcmA repeats to the pathogenic bacterium present in the blood. A "flag" protein provided by the invention will then activate a memory response, i.e. the response to the antigenic determinant present in the protein. The antibodies thus produced recognise the "flag"- labelled bacteria, which will then be neutralised by the immune system. In this way the protein is used to stimulate a pre-existing (memory) immune response, non-related to the bacterial infection, to clear bacterial infections from the system. Yet another use (which alternatively may be considered medical use or food use) provided by the invention is the use wherein a protein provided by the invention has the ability to bind to cells, such as mucosal cells, e.g. of the gut. The reactive group of such a protein is in such a case for example partly or wholly derived from a fimbriae protein or another gut attachment protein, as for example present in various E. *coli* strains. Micro-organisms to which such a protein is attached will specifically home or bind to certain areas of the gut, a property which for example is beneficial for certain bacterial strains (i.e. lactococcal or lactobacillar strains) used as a probiotic. In another food or use of food provided by the invention, the protein or substance provided by the invention

is a composed of a food additive (such as an enzyme or flavour compound) which affects quality, flavour, shelf-life, food value or texture, joined with an attaching peptide, and subsequently attached or anchored to a micro-organism which is than mixed with the foodstuff. The anchoring of such proteins to a bacterial carrier offers the additional advantage that the additive can be targeted to a solid (bacteria-containing) matrix (e.g. curd) in a process for the preparation of food, e.g. cheese or tofu. Yet another use of a proteinaceous substance or micro-organism provided by the invention is the use of bacterial surface display in generating whole-cell bioadsorbents or biofilters for environmental purposes, microbiocatalysts, and diagnostic tools

The invention is further explained in the experimental part which can not be seen as limiting the invention.

Experimental part

Introduction

The major autolysin AcmA of *Lactococcus lactis* subsp. *cremoris* MG1363 is an *N*-acetylmuramidase which is required for cell separation and is responsible for cell lysis during stationary phase (5, 6). The 40.3-kDa secreted mature protein produces a number of activity bands in a zymogram of the supernatant of a lactococcal culture. Bands as small as that corresponding to a protein of 29 kDa were detected. As no clearing bands are produced by an *L. lactis acmA* deletion mutant, all bands represent products of AcmA (6). From experimental data and homology studies we inferred that AcmA likely consists of three domains: a signal sequence followed by an active site domain and a C-terminal region containing three highly homologous repeats of approximately 45 amino acids which are involved in cell wall binding. As the smallest active protein is 29 kDa, it was suggested that the protein

undergoes proteolytic breakdown in the C-terminal portion (5, 6).

Cell wall hydrolases of various bacteria and bacteriophages contain repeats similar to those present in AcmA (4, 9, 10, 17). Partially purified muramidase-2 of *Enterococcus hirae*, a protein similar to AcmA, containing 6 similar repeats, binds to peptidoglycan fragments of the strain (11). The p60 protein of *Listeria monocytogenes* contains two such repeats and was shown to be associated with the cell surface (24). However, which parts of these enzymes contained the binding capacity was not assessed in any of these studies.

Nearly all cell wall hydrolases examined so far seem to consist of a catalytic domain and usually, although not always, a domain containing a number of specific amino acid repeats. In several studies it has been shown that only a part of some of the cell wall hydrolases is required for enzymatic activity (13, 14, 17, 19, 22, 34). Rashid *et al.* reported the cloning of the gene encoding a 90-kDa glucosaminidase of *Bacillus subtilis* of which the C-terminus shows significant similarity with the glucosaminidase domain of the *S. aureus* autolysin (23). The protein contains two repeated sequences in its N-terminus and two different repeats in the middle domain. A deletion derivative lacking the C-terminal 187 amino acids remained tightly bound to the cell walls, but no catalytic activity was observed when expressed in *B. subtilis*. By making deletions from the N-terminus it was shown that nearly two-thirds of the protein could be removed without complete loss of cell wall-hydrolyzing activity in *E. coli*, although loss of more than one repeat drastically reduced lytic activity.

The N-terminal domain of the major autolysin LytA of *Streptococcus pneumonia* provides the *N*-acetylmuramyl-L-alanine amidase catalytic function, whereas the C-terminal domain, which contains six repeated sequences, determines the specificity of binding to the cell wall (for review: see

reference 18). The protein lacks a signal sequence and requires choline-containing teichoic acids to fully degrade pneumococcal cell walls. Furthermore, it was shown that at least four of the six repeats were needed for efficient recognition of the choline residues of pneumococcal cell walls and the retention of appreciable hydrolytic activity (7).

LytA, pneumococcal phage lysins as well as clostridial and lactococcal cell wall hydrolases have been used for the construction of active proteins, such that the activity domain and cell wall recognition domains were exchanged. The N-terminal half of the lactococcal phage enzyme was fused to the C-terminal domain of LytA (28). The chimeric enzyme exhibited a glycosidase activity capable of hydrolyzing choline-containing cell walls of *S. pneumonia*. This result showed that the lactococcal phage lysin consisted of at least two domains with a glucosidase activity contained in its N-terminus and two repeats similar to those in AcmA in the C-terminus (6). A tripartite pneumococcal peptidoglycan hydrolase has been constructed by fusing the N-terminal catalytic domain of the phage CPL1 lysozyme to HBL3, a protein with an amidase activity and a choline-binding domain (27). The three domains acquired the proper conformation as the fusion protein behaved as an amidase, a lysozyme and as a choline-dependent enzyme.

Also from nature an enzyme is known having two separate functional activity domains: the autolysin gene from *Staphylococcus aureus* encodes a protein that contains an amidase and an endo-β-*N*-acetylglucosaminidase domain separated by three highly similar repeats (20). This protein is processed posttranslationally into the two constituting activity domains.

The aim of the present study was to investigate the modular structure of AcmA. This was done by consecutively deleting the C-terminal repeats and by fusing the repeats to heterologous proteins. On the basis of cell fractionation and binding studies involving whole cells, it is concluded that

the C-terminal repeats in AcmA bind the autolytic enzyme to the cell wall of *L. lactis*.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *Lactococcus lactis* was grown at 30°C in two-fold diluted M17 broth (Difco Laboratories, Detroit, Mich.) containing 0.5% glucose and 0.95% β-glycerophosphate (Sigma Chemical Co., St. Louis, Mo.) as standing cultures (½M17). Agar plates of the same medium contained 1.5% agar. Five μg/ml of erythromycin (Boehringer GmbH, Mannheim, Germany) was added when needed. *Escherichia coli* was grown at 37°C with vigorous agitation in TY medium (Difco), or on TY medium solidified with 1.5% agar. When required, the media contained 100 μg of ampicillin (Sigma), 100 μg erythromycin or 50 μg kanamycin (both from Boehringer) per ml. Isopropyl-ß-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (both from Sigma) were used at concentrations of 1 mM and 0.002%, respectively.

General DNA techniques and transformation.

Molecular cloning techniques were performed essentially as described by Sambrook *et al.*(25). Restriction enzymes, Klenow enzyme and T4 DNA ligase were obtained from Boehringer and were used according to the instructions of the supplier. Deoxynucleotides were obtained from Pharmacia (Pharmacia Biotech, Uppsala, Sweden). All chemicals used were of analytical grade and were from Merck (Darmstadt, Germany) or BDH (Poole, United Kingdom). Electrotransformation of *E. coli* and *L. lactis* was performed by using a gene pulser (Bio-Rad Laboratories, Richmond, Calif.), as described by Zabarovsky

and Winberg (37) and Leenhouts and Venema (16), respectively. Plasmid DNA was isolated using the QIAGEN plasmid DNA isolation kit (QIAGEN GmbH, Hilden, Germany) or by CsCl-ethidiumbromide density gradient centrifugation and DNA fragments were isolated from agarose gels using the QIAGEN gel extraction kit and protocols from QIAGEN.

Primer synthesis, PCR and DNA Sequencing.

Synthetic oligo deoxyribonucleotides were synthesized with an Applied Biosystems 392 DNA/RNA synthesizer (Applied Biosystems Inc., Foster City, Calif.). The sequences of the oligonucleotides used are listed in Table 2.

Polymerase chain reactions (PCR) were performed in a Bio-Med thermocycler 60 (Bio-Med GmbH, Theres, Germany) using super *Taq* DNA polymerase and the instructions of the manufacturer (HT Biotechnology Ltd., Cambridge, United Kingdom). PCR fragments were purified using the nucleotide removal kit and protocol of QIAGEN.

Nucleotide sequences of double-stranded plasmid templates were determined using the dideoxy chain termination method (26) with the T7 sequencing kit and protocol (Pharmacia) or the automated fluorescent DNA sequencer 725 of Vistra Systems (Amersham Life Science Inc., Buckinghamshire, United Kingdom).

Nucleotide and amino acid sequences were analyzed with the PC/GENE sequence analysis program (version 6.8. IntelliGenetics, Inc., Geneva, Switzerland). Protein homology searches in the SWISSPROT, PIR, and Genbank (release September 23, 1996) databases were carried out with the BLAST program (1).

Construction of AcmA derivatives.

A stop codon and *Eco*RI restriction enzyme site were introduced in *acmA* at the end of nucleotide sequences encoding the repeats and at the end of the sequence specifying the active site domain by PCR using the primers REPDEL-1, REPDEL-

2, and REPDEL-3 and plasmid pAL01 as a template. Primer ALA-4, annealing within the sequence encoding the signal peptide of AcmA, was used in all cases as the upstream primer. All three PCR products were digested with *Sac*I and *Eco*RI and cloned into the corresponding sites of pBluescript SK+ leading to pDEL1, pDEL2, and pDEL3. Subsequently, the 1,187-bp *Pflm*I-*Eco*RI fragment of pGKAL1 (5) was replaced by the 513, 282 and 76-bp *Pflm*I-*Eco*RI fragments of the inserts of pDEL1, 2 and 3, respectively. The proper plasmids specifying proteins containing one, two or all three repeats (pGKAL5, 4, and 3, respectively) were obtained in *L. lactis* MG1363*acmAΔ1*. pGKAL1 was cut with *Spe*I. The sticky ends were flushed with Klenow enzyme and self-ligation introduced a UAG stop codon after the Ser 339 codon of *acmA*. The resulting plamid was named pGKAL6.

A DNA fragment encoding half of the first repeat until the *Spe*I site in the middle of the second repeat was synthesized by PCR using the primers REP-4 A and B. The *Nhe*I and *Spe*I sites at the ends of the 250-bp PCR product were cut and the fragment was cloned into the unique *Spe*I site of pGKAL1 resulting in plasmid pGKAL7.

Overexpression and isolation of the AcmA active site domain.

A DNA fragment encoding the active site domain of AcmA was obtained using the primers ACMHIS and REPDEL-3 with plasmid pAL01 as a template. The 504-bp PCR fragment was digested with *Bgl*II and *Eco*RI and subcloned into the *Bam*HI and *Eco*RI sites of pET32A (Novagen R&D systems Europe Ltd, Abingdon, United Kingdom). The proper construct, pETAcmA, was obtained in *E. coli* BL21(DE3) (30). Expression of the thioredoxin/AcmA fusion protein was induced in this strain by adding IPTG (to 1 mM final concentration) at an OD_{600} of 0.7. Four hours after induction the cells from 1 ml of culture were collected by centrifugation and the fusion protein was purified over a

Talon™ metal affinity resin (Clontech Laboratories Inc., Palo Alto, Calif.) using 8 M ureum-elution buffer and the protocol of the supplier. The eluate (200 µl) was dialyzed against a solution containing 50 mM NaCl and 20 mM Tris (pH 7) after which $CaCl_2$ was added to a final concentration of 2 mM. One unit of enterokinase (Novagen) was added and the mixture was incubated at room temperature for 20 h. The protein mixture was dialyzed against several changes of demineralized water before SDS-PAGE analysis and cell binding studies.

Construction of β-lactamase and α-amylase fusions to the AcmA repeat domain.

For the introduction of a unique *Nde*I site at the position of the stop codon of the *E. coli* TEM-β-lactamase, the oligonucleotides BETA-1 and BETA-2 were used in a PCR with plasmid pGBL1 (21) as a template. The 403-bp PCR fragment was cut with *Nde*I and *Pst*I and cloned as a 311-bp fragment into the same sites of pUK21. The resulting plasmid, pUKblac, was digested with *Nde*I, treated with Klenow enzyme and subsequently digested with *Xba*I. The β-lactamase encoding fragment was ligated to an 1,104-bp *Pvu*II-*Xba*I DNA fragment from pAL01 containing the *acmA* part encoding the repeat region of AcmA. The resulting plasmid, pUKblacrep, was digested with *Pst*I and *Dra*I and the 1349-bp fragment was inserted into the *Pst*I-*Sna*BI sites of pGBL1, leading to plasmid pGBLR. After digestion of pGAL9 (21) with *Cla*I and *Hin*dIII the 1,049-bp fragment encompassing the 3'-end of the *Bacillus licheniformis* α-amylase gene was subcloned into corresponding sites of pUK21. According to the paper of Pérez Martínez *et al.* (21), this fragment should be 1,402-bp, but after restriction enzyme analysis it turned out to be approximately 350-bp smaller. The resulting plasmid was called pUKAL1. A unique *Eco*RV restriction enzyme site was introduced by PCR at

the position of the stop codon of the *B. licheniformis* α-amylase gene using the oligonucleotides ALFA-A and ALFA-B with plasmid pGAL9 as a template. After restriction of the 514-bp PCR fragment with *Sal*I and *Eco*RV the 440-bp fragment was cloned into the same sites of pUKAL1 resulting in plasmid pUKAL2. The *Eco*RV and *Xba*I sites of this plasmid were used to clone the 1,104-bp *Pvu*II-*Xba*I fragment of pAL01 encoding the repeats of AcmA. The 1,915-bp *Cla*I-*Hin*dIII fragment of the resulting plasmid pUKALR was used to replace the corresponding 1,049-bp fragment of pGAL9 (pGALR). All cloning steps described above were performed in *E. coli* NM522. The plasmids pGBL1, pGBLR, pGAL9 and pGALR were used to transform *L. lactis* MG1363 and MG1363*acmAΔ1*.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and detection of AcmA and α-amylase activity.

Two ml of end exponential phase *L. lactis* cultures were subjected to centrifugation. 0.5 ml of the supernatant fractions were dialyzed against several changes of demineralized water, lyophilized, and dissolved in 0.25 ml of denaturation buffer (3). Cell pellets were washed with 2 ml of fresh ½M17 medium and resuspended in 1 ml of denaturation buffer. Cell extracts were prepared as described by van de Guchte *et al.*(32).

AcmA activity was detected by a zymogram staining technique using SDS-PAA (12.5% or 17.5%) gels containing 0.15% autoclaved, lyophilized *Micrococcus lysodeikticus* ATCC 4698 cells (Sigma) as described before (6). For the analysis of α-amylase activity 1% starch was included into 12.5% PAA gels. After electrophoresis proteins were renatured using the AcmA renaturation solution (3) and the gel was stained with an I_2/KI solution (at final concentrations of 12 and 18 mM, respectively) (33).

SDS-PAGE was carried out according to Laemmli (15) with the Protean II Minigel System (Bio-Rad) and gels were stained with Coomassie brilliant blue (Bio-Rad). The standard low range and prestained low and high range SDS-PAGE molecular weight markers of Bio-Rad were used as references.

Fractionation of mid- and end-exponential phase cultures of *L. lactis* was performed according to the protocol of Baankreis (2).

Binding of AcmA and its derivatives to lactococcal cells.

The cells of 2 ml of exponential phase cultures of MG1363*acmAΔ1* were gently resuspended in an equal volume of supernatant of similarly grown MG1363*acmAΔ1* carrying either plasmid pGK13, pGKAL1, -3, -4, -5, -6 or -7 and incubated at 30°C for 20 min. Subsequently, the mixtures were centrifugated.

The cell pellets were washed with 2 ml of ½M17 and cell extracts were prepared in 1 ml of denaturation buffer as described above, while 0.4 ml of the supernatants were dialyzed against demineralized water, lyophilized and dissolved in 0.2 ml of denaturation buffer.

To analyze competitive binding between AcmA derivatives containing 1 or 2 repeats, equal volumes of the supernatants of MG1363*acmAΔ1* containing pGKAL3 or pGKAL4 were mixed prior to incubation with the MG1363*acmAΔ1* cells. The samples were treated for SDS-PAGE as described above.

Three 500 µl samples of a mid-exponential phase culture of MG1363*acmAΔ1* were centrifugated. From one sample 50 µl of the supernatant were replaced by 50 µl of a solution containing the AcmA active site domain (see above). 100 µl of the supernatant of sample two were replaced by 50 µl demineralized water and 50 µl of the supernatant of a mid-exponential phase culture of MG1363*acmAΔ1* (pGKAL4). Of the third sample 100 µl of the supernatant were replaced by 50 µl of the solution containing the AcmA active site domain and 50 µl of the supernatant of MG1363*acmAΔ1* (pGKAL4). Subsequently the three samples were vortexed to resuspend the cells and incubated for 15 min at 30°C. After centrifugation cell and supernatant fractions were prepared in 500 µl of denaturation buffer for analysis of AcmA activity in SDS-(17.5%)PAGE as described above.

Binding of the β-lactamase/AcmA fusion protein was studied by growing MG1363*acmAΔ1* containing pGK13, pGBL1 or pGBLR until mid-exponential phase. The cells of 1 ml of MG1363*acmAΔ1*(pGK13) culture were resuspended in an equal volume of supernatant of either of the other two cultures. The mixtures were prepared in duplo and one series was incubated at 30°C for 5 min while the other was kept at that temperature

for 15 min. Then, cell and supernatant fractions were treated as described for the AcmA binding studies, resuspended in denaturaion buffer in half of the original volume, and subjected to SDS-(12.5%)PAGE followed by Western blot analysis.

Western blotting and immunodetection.

Proteins were transferred from SDS-PAA gels to BA85 nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) as described before (31). α-amylase and β-lactamase antigen was detected with 2000-fold diluted rabbit polyclonal anti-ampicillinase antibodies (5 prime→3 prime, Inc., Boulder, Co.), and alkaline phosphatase-conjugated goat anti-rabbit antibodies (Promega Corporation, Madison, Wis.) using the Western-Light Chemiluminescent detection system and protocol (TROPIX Inc., Bedford, Mass.).

Enzyme assays and optical density measurements.

AcmA activity was visualized on ½M17 agar plates containing 0.2% autoclaved lyophilized *M. lysodeikticus* cells as halo's around colonies after overnight growth at 30°C.

α-amylase activity was detected by spotting 10 µl of an overnight culture onto a ½M17 agar plate containing 1% of starch (Sigma). After 18 h of incubation at 30°C halo's were visualized by staining with an iodine solution according to the protocol of Smith *et al.* (29). A similar method was used for the detection of β-lactamase activity (29).

X-prolyl dipeptidyl aminopeptidase (PepX) was measured using the chromogenic substrate Ala-Pro-*p*-nitroanilid (BACHEM Feinchemicalien AG, Bubendorf, Switserland). After 2 min of centrifugation in an eppendorf microcentrifuge 75 µl of a culture supernatant was added to 50 µl substrate (2 mM) and 75 µl Hepes buffer (pH 7). The mixture was pipetted into a microtiter plate well and colour development was monitored in a THERMOmax microtiter plate reader (Molecular Devices

Corporation, Menlo Oaks, Ca) at 405 nm during 20 minutes at 37°C. Optical densities were measured in a Novaspec II spectrophotometer (Pharmacia) at 600 nm.

Results

Two of the three repeats in AcmA are sufficient for autolysis and cell separation.

Several mutant AcmA derivatives were constructed to investigate the function of the three repeats in the C-terminus of AcmA. A stop codon was introduced behind the codon for Thr-287 (pGKAL4) or Ser-363 (pGKAL3) (see Fig. 1). Plasmid pGKAL4-specified AcmA (A1) only contains the first (most N-terminal) of the three repeats, while pGKAL3 specifies an AcmA variant (A2) carrying the first two repeats. pGKAL5 specifies an AcmA derivative lacking repeats (A0) due to the introduction of a stop codon after Ser-218. AcmA specified by pGKAL6 contains one an a half repeat (A1.5) due to the presence of a stop codon behind the Ser-339 codon. From pGKAL7 an AcmA mutant (A4) is produced which carries an additional (fourth) repeat as the result of duplication of the polypeptide from Ser-263 to Thr-338. All proteins were expressed from the *acmA* promoter in the AcmA-negative strain *L. lactis* MG1363*acmAΔ1*. The various deletions of AcmA were examined with respect to the following properties: (I) their effect on halo formation on plates containing cell wall fragments of *M. lysodeikticus*, (II) chain length of the cells expressing the mutant AcmA's, and sedimentation of the cells in a standing culture, (III) their enzymatic activity, both in the cell and supernatant fraction and (IV) autolysis.

Halo formation. On a ½M17 plate containing cell wall fragments of *M. lysodeikticus* halo's were absent when MG1363*acmAΔ1* carried pGK13 or pGKAL5. All other strains produced a clear halo that differed in size. The halo size was

clearly correlated with the number of full length repeats present, although the addition of an extra repeat resulted in a reduced halo size (see Table 3). Apparently, for optimal cell wall lytic activity a full complement of repeats is required.

Cell separation and sedimentation. The deletion of one and a half, two and all three repeats had a clear effect on the chain length and on sedimentation of the cells after overnight growth (see Table 3). Thus, efficient cell separation requires the presence of at least two repeats in AcmA.

Enzyme activity. Cells and supernatants of overnight cultures of all strains were analyzed for AcmA activity by SDS-PAGE. In the cell fractions no activity was detected for A0, not even after one week of renaturation of the protein (Table 3). Of the other derivatives, two major activity bands were present in this fraction. In each case their positions in the gel corresponded to proteins with the calculated molecular weights of the unprocessed and the processed form. (Table 3 and not shown). As shown in Fig. 1, all AcmA derivatives were still active in the supernatant fractions. AcmA produced the characteristic breakdown pattern as determined before (Fig. 1, lanes 1 and 3; (6)). All AcmA derivatives except A0 and A1 also showed a distinct and highly reproducible degradation pattern. A4 showed 2 additional breakdown products after prolonged renaturation (results not shown). These data indicate that removal of the repeats does not destroy AcmA activity and suggests that one repeat is sufficient to keep the enzyme cell-associated.

Autolysis. To analyze the effect of the repeats on autolysis during stationery phase, overnight cultures of all strains were diluted hundred-fold and incubated at 30°C for 6 days and the decrease of optical density (OD_{600}) was followed. All cultures exhibited similar growth rates, reached the same maximal optical densities and did not lyse during the

exponential phase of growth. After approximately 60 h of incubation maximal reduction in OD_{600} was reached in all cases. The results are presented in Table 3 and show that the reduction in OD_{600} is correlated with the reduction of the number of AcmA repeats. To investigate whether the decrease in OD_{600} really reflected autolysis, the activity of the intracellular enzyme PepX was measured. After 60 h of incubation, PepX activity in the culture medium was also maximal in all samples, decreasing in all cases upon further incubation. Hardly any PepX activity was detected in the supernatant of the *acmAΔ1* mutant and in cultures producing A0, A1 or A1.5. In contrast, a considerable quantity of PepX had released into the supernatant of cultures producing A2 and A3. Thus two repeats in AcmA are sufficient for autolysis of *L. lactis*. A2 or A4 production led to reduced lysis of the producer cells. Taken together, these results indicate that the repeats in AcmA function in efficient autolysis and are required for cell separation.

The active site domain of AcmA resides in the N-terminal part.

To examine whether the active site is located in the N-terminal domain of AcmA, a DNA fragment starting at codon 58 until codon 218 of *acmA* was synthesized by PCR and fused to the thioredoxin gene in plasmid pET32A. The fusion protein comprises 326 amino acids. A protein with the expected molecular mass (35 kDa) was isolated from a culture of *E. coli* BL21(DE3) (pETAcmA) (Fig. 2, lane A2). By cleavage with enterokinase, the protein was split into a thioredoxin part of 17 kDa and an AcmA domain (nA) of 18 kDa (Fig. 2, lane A1). The zymogram (Fig. 2.B) shows that the fusion protein did not have appreciable cell wall hydrolytic activity, while the released domain of AcmA was active (Fig. 2, lanes B1 and B2), indicating that the active site domain was in the N-terminal part of AcmA.

Fusion of the repeats of AcmA to α-amylase and β-lactamase yields active enzymes.

The three C-terminal repeats of AcmA (cA) were fused C-terminally to *B. licheniformis* α-amylase and *E. coli* TEM β-lactamase as described in Material and Methods and shown in Fig. 3. The hybrid proteins were fused to the lactococcal signal sequences AL9 and BL1, respectively (21). Both fusion proteins were active in plate assays, as is only shown for the β-lactamase/AcmA fusion protein (βcA) (Fig. 4). The halo's around colonies producing the fusion proteins were smaller than those produced by the wild-type enzymes, which could either be caused by reduced intrinsic enzyme activities due to the presence of repeats or by increased susceptibility to proteolytic degradation. However, the smaller halo's produced by the chimeric proteins might also be caused by hampered diffusion due to cell wall binding (see below).

The activities of α-amylase and the αcA fusion protein were also detected in a renaturing SDS-(12.5%)PAA gel containing 1% starch. The primary translation product of the α-amylase gene is a protein of 522 amino acid residues which contains a signal sequence of 37 amino acids (21). It is secreted as a 55-kDa protein. αcA consists of 741 amino acids and, if processed and secreted, would give rise to a 78-kDa protein. Cell and supernatant fractions of *L. lactis* MG1363 and MG1363*acmAΔ1* carrying pGAL9 or pGALR were analyzed after overnight growth of the strains. The results are presented in Fig. 5 and show that the clearing bands are present at the position expected for both mature proteins. Apparently, αcA is active. Clearly, smaller products are present in the supernatants of the cells producing the fusion protein, the smallest being approximately of the size of wild-type mature α-amylase (Fig. 5 and not shown).

The β-lactamase fusion protein is predominantly present in the cell wall.

To examine whether the presence of the C-terminal domain of AcmA resulted in binding of βcA to the cell wall, mid-exponential phase cultures of *L. lactis* MG1363*acmAΔ1* containing pGBL1, encoding β-lactamase, or pGBLR, specifying βcA, were fractionated and subjected to Westen blot analysis (Fig. 6). From pGBL1, β-lactamase is expressed as a protein of 322 amino acids containing a signal sequence of 47 amino acids. The secreted protein is 30 kDa. βcA consists of 540 amino acids and is secreted as a protein with a molecular mass of 52 kDa. Figure 6 shows that most of the wild-type β-lactamase is present in the culture supernatant and none in the cytoplasm. Slightly larger bands, likely representing the unprocessed form, are found in the membrane fractions of this strain. In contrast, βcA is predominantly retained in the cell wall fraction, although a considerable amount resides in the cytoplasm, strongly suggesting that the AcmA repeats anchored the hybrid enzyme to the cell wall. The smaller band present in both cytoplasmic fractions is caused by cross hybridization of the antibodies to an unspecified lactococcal protein (unpublished observation). In the supernatant fraction of cells producing βcA, only little full length protein was observed. Several distinct smaller products are present in this fraction which were also detectable in very low amounts in the cell wall fraction after prolonged exposure of the film (not shown) but were absent from the other fractions.

The C-terminal repeats in AcmA are required for cell wall binding.

Although the results presented in the previous section strongly suggests that the C-terminal repeats are required for the retention of protein in the cell wall, definite proof was obtained by mixing the supernatant fractions of end-exponential phase cultures containing AcmA, or one of its

deletion derivatives (see Fig. 1), with the cells from an equal volume of a culture of MG1363*acmAΔ1* (pGK13). After incubation, cell and supernatant fractions were examined for the presence of AcmA. Except for A0, all proteins were capable of binding to the MG1363*acmAΔ1* cells (Table 3). Also, all degradation products of AcmA and its derivatives were capable of binding. The finding that A0 was unable to bind was corroborated by adding the mixture of enterokinase-released nA and thioredoxin to supernatant containing A1. When incubated with AcmA-minus cells, only A1 bound to the lactococcal cells (Fig. 7) as only this protein was detectable in the cell fraction. nA was only detected in the supernatant. This was also the case when the experiment was repeated with nA alone (not shown).

Binding of AcmA or βcA to lactococcal cells at different pHs.

The supernatant fraction of a mid-exponential phase *L. lactis* MG1363*acmAΔ1* culture was replaced by the supernatant of a mid-exponential phase *L. lactis* MG1363 culture. This mixture was incubated at 30°C for 5 min. Thereafter the supernatant was removed by centrifugation and the cell pellet was washed with M17. The cell pellets were dissolved in M17 with pHs ranging from 2 to 10 and incubated at 30°C for 30 min. The cell and supernatant fractions were separated and treated as described before and analysed for the presence of AcmA activity. A similar experiment was executed with mid-exponential phase *L. lactis* MG1363*acmAΔ1* cells with the supernatant of an *L. lactis* MG1363*acmAΔ1*(pGBLR) culture. The presence of βcA was analysed by western blotting and immunodetection as described.

At all different pHs, AcmA and βcA was found to be bound to the lactococcal cells. The binding of both AcmA and βcA was better at low pH as judged from the activity in a zymogram and

the visual presence of the amount of βcA fusion protein in the cell extracts after immunodetection.

Proteolytic breakdown of AcmA by Pronase and Trypsin.

The supernatant fraction of a mid-exponential phase MG1363*acmAΔ1* culture was replaced by the supernatant of a mid-exponential phase MG1363 culture. This mixture was incubated at 30°C for 15 min. Thereafter the supernatant and the cell fractions were separated and the cell pellet was dissolved in an identical volume of M17. To both fractions Pronase and Trypsin (1 mg/ml) dissolved in 10 mM NaPi buffer (pH=7) was added to an end concentration of (10 μg/ml) and the mixtures were incubated at 30°C. Samples were taken after 5 and 30 min and 2 h of incubation. The cell and supernatant fractions of each sample were separated and prepared for zymographic analysis as described above.

A complete hydrolysis of AcmA by pronase was observed in the supernatant fraction after 2 h of incubation while activity was still present in the cell extract at this time point. The hydrolysis of AcmA by trypsin was slower and activity was still present in the supernatant after 2 h of incubation. In time the portion of activity present in the cell extracts was always higher than that observed in the supernatant. These results indicate that the AcmA protein is protected when it is bound to the cell.

Binding of AcmA to different types of bacterial cells.

The strains *Bacillus subtilis* DB104, *Lactobacillus plantarum* 80, *Streptococcus faecalis* JH2-2, *Streptococcus thermophilus* ATCC 19258, *Listeria P*, *Lactobacillus buchneeri* L4, *Clostridium beijerinckii* CNRZ 530 and *Escherichia coli* NM522 were grown overnight in GM17. Two fractions of each overnight culture were centrifuged and the supernatants were replaced by the supernatant of an overnight-culture of *L.*

lactis MG1363*acmAΔ1*(pGKAL1) or MG1363*acmAΔ1*(pGK13). The mixtures were incubated at 30°C for 15 min. Subsequently the cell and supernatant fractions were separated and the cells were washed once with M17 and were prepared for SDS-PAGE as described before and analysed for AcmA activity.

In all cell extracts AcmA activity was present while such an activity was absent in extracts of cells which had been incubated with the supernatant of MG1363*acmAΔ1*(pGK13) which lacks the presence of AcmA.

To investigate the effect of repeat number on binding, equal volumes of the supernatants of cultures of MG1363*acmAΔ1* (pGKAL3, encoding A2) and MG1363*acmAΔ1* (pGKAL4, specifying A1) were mixed. The undiluted and a 10-fold diluted mixture were incubated with the AcmA-free cells. Analysis of zymograms of serial dilutions showed that the two activities were equally distributed over the cell and supernatant fractions, indicating that both proteins bind equally well (results not shown).

To examine whether the C-terminal repeat sequences of AcmA had the capacity to bind a heterologous, extracellular enzyme to lactococcal cells, binding of βcA was assessed by incubation of AcmA-minus *L. lactis* cells with culture supernatants containing either secreted wild-type β-lactamase or βcA. As Fig. 8 shows, wild-type β-lactamase was exclusively present in the supernatant fraction, whereas βcA fractionated with the lactococcal cells and, thus, had bound to these cells.

Discussion

The results presented in this work indicate that the mature form of the *N*-acetylmuramidase AcmA of *L. lactis* consists of two separate domains. The overproduced and purified N-terminus, from amino acid residue 58 to 218 in the pre-protein, is active on *M. lysodeikticus* cell walls and, thus, contains the active site of the enzyme. This is in agreement with the finding that the repeat-less AcmA mutant A0 can still hydrolyze *M. lysodeikticus* cell walls, albeit with severely reduced efficiency. Prolonged renaturation was needed to detect the activity of the enzyme *in vitro* while colonies producing the protein did not form a halo. Enzymes A1 and A2 had *in vitro* activities which were nearly the same as that of the wild-type protein, although in the plate assay A1 produced a smaller halo than A2 which, in turn, was smaller than the wild-type halo. A strain producing A1 grew in longer chains than cells expressing A2 and, in contrast to A2 producing cells, sedimented and did not autolyze. Taken together these results indicate that, although the N-terminus of AcmA contains the active site, the presence of at least one complete repeat is needed for the enzyme to retain appreciable activity. Second, only cultures producing AcmA's containing two or more full length repeats are subject to autolysis and produce wild-type chain lengths. It is tempting to speculate that this apparent increase in catalytic efficiency of AcmA is caused by the repeat domain by allowing the enzyme to bind to its substrate, the peptidoglycan of the cell wall. As was postulated by Knowles *et al.* (12) for the cellulase binding domains in cellobiohydrolases, such binding would increase the local concentration of the enzyme. The repeats could be involved in binding alone or could be important for proper positioning of the catalytic domain towards its substrate. The increase in AcmA activity with an increasing number of repeats to up to 3 in the wild-type enzyme, suggests an evolutionary

process of repeat amplification to reach an optimum for proper enzyme functioning. The binding of A1, A1.5 and A4 was comparable with that of wild-type AcmA but these enzyme varieties caused no or only little autolysis. These observations seem to support the idea that 3 repeats are optimal for proper functioning of AcmA. The presence of 5 and 6 repeats in the very similar enzymes of *E. faecalis* and *E. hirae*, respectively, may reflect slight differences in cell wall structure and/or the catalytic domain, requiring the recruitment by these autolysins of extra repeats for optimal enzyme activity.

The hypothesis that the C-terminal domain of AcmA is involved in cell binding (6) was corroborated in this study. First of all we show that AcmA is indeed capable of cell binding. AcmA and its derivatives A1, A1.5, A2 and A4 all bound to cells of *L. lactis* when added from the outside. To prove that it was the C-terminus of AcmA that facilitated binding and not some intrinsic cell wall binding capacity of the N-terminal domain, the repeat domain was fused to two heterologous proteins which do not normally associate with the cell wall. The smaller halo's produced by αcA and βcA compared to the wild-type proteins and the presence of most of βcA in the cell wall fraction are indicative of cell binding of the fusion proteins via the AcmA-specific repeats.

The βcA binding studies clearly show that it is the AcmA repeat domain that specifies cell wall binding capacity: whereas wild-type β-lactamase (and, for that matter, repeat-less AcmA) did not bind to lactococcal cells, βcA did bind to these cells when added from the outside. The results obtained with A1 in the binding assay show that only one repeat is sufficient to allow efficient binding of AcmA. In a separate study (5) we showed that AcmA can operate intercellularly: AcmA-free lactococcal cells can be lyzed when grown together with cells producing AcmA. Combining this observation with the results presented above allows to conclude that AcmA does not

only bind when confronting a cell from the outside but, indeed, is capable of hydrolyzing the cell wall with concomitant lysis of the cell.

AcmA-like repeats were found to be present at different locations in more than 30 proteins after a comparison of the amino acid sequences of the repeats in AcmA with the protein sequences of the Genbank database (release 23). Not all of these proteins with repeats varying from one to six are cell wall hydrolases. Alignment of the amino acid sequences of all the repeats yielded a consensus sequence similar to that postulated by Birkeland and Hourdou *et al.* (4, 9). Interestingly, if a limited number of modifications are allowed in the consensus repeat, the repeat is also present 12 and 4 times, respectively, in two proteins of *Caenorhabditis elegans*, which both show homology with endochitinases (Gene accession numbers U64836 and U70858) (36). Possibly, these repeats anchor these enzymes to fungi ingested by this organism. The presence of similar repeats in proteins of different bacterial species strongly suggests that they recognize and bind to a general unit of the peptidoglycan. An interesting goal for the future will be to elucidate the unit to which they bind and the nature of the binding.

As has been reported earlier for intact AcmA (5), and, as we show here for its C-terminal deletion derivatives, the enzyme is subject to proteolytic degradation. None of the degradation products were present in cell extracts of whole cells indicating that they are not formed inside the cell (data not shown). The degradation pattern of each AcmA derivative is specific and very reproducible. Based on the sizes of the degradation products, a number of the proteolytic cleavage sites probably resides in the intervening sequences. One such site (1 in Fig. 1) is present between repeat 1 and 2. Cleavage at this position would result in an active protein of approximately 28 kDa, which is indeed seen in the supernatants of all strains producing AcmA with 1.5 or more repeats. A

second cleavage site is probably located between the second and third repeat (2 in Fig. 1). Cleavage at this site is either rather infrequent, or the resulting degradation product is not very active, which, in both cases, would lead to the faint bands of activity observed in lanes 1 and 3 of the zymogram presented in Fig. 1. The presence of cleavage sites in between the AcmA repeats is further suggested by the presence of specific degradation products observed in αcA and βcA; their sizes are in accord with the location of the cleavage sites postulated in AcmA. In addition, as also bands of the size of the wild-type α-amylase and β-lactamase are observed, an additional cleavage site seems to be present around the fusion point of these enzymes and the cell wall binding domain of AcmA.

All degradation products of AcmA and those of the two fusion proteins are mainly present in the supernatant and to some extent in the cell wall fraction, but not in the cells. As none of the *L. lactis* strains used produced the cell wall-anchored proteinase PrtP, this enzyme can not be held responsible for the specific degradation of AcmA or the fusion proteins. Apparently, an extracellular proteinase exists in *L. lactis* that is capable of removing the repeats, which may represent a mechanism for the regulation of AcmA activity.

TABLE 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant phenotype(s) or genotype(s)	Source or reference
Strains		
L. lactis subsp. *cremoris*		
MG1363	Plasmid-free strain	(8)
MG1363*acmAΔ1*	Derivative of MG1363 carrying a 701-bp *Sac*I-*Spe*I deletion in *acmA*	(6)
E. coli		
NM522	*sup*E *thi* Δ(*lac-proAB*) Δ*hsd*5(r_k^-,m_k^-)[F' *proAB lacI*^{q}*ZM15*]	Stratagene
BL21(DE3)	*ompT* $r_B^-m_B^-$ *int*; bacteriophage DE3 lysogen carrying the T7 RNA polymerase gene controlled by the *lac*UV5 promoter	(30)
Plasmids		
pET32A	Ap^r, vector for high level expression of thioredoxin fusion proteins	Novagen
pUK21	Km^r, general cloning vector	(35)
pBluescript SK+	Ap^r, general cloning vector	Stratagene
pAL01	Ap^r, pUC19 carrying a 4,137-bp lactococcal chromosomal DNA insert with *acmA* gene	(6)
pDEL1	Ap^r, pBluescript SK+ with 785-bp *Sac*I-*Eco*RI fragment of *acmA* obtained by PCR with primers ALA-4 and REPDEL-1	This work
pDEL2	Ap^r, pBluescript SK+ with 554-bp *Sac*I-*Eco*RI fragment of *acmA*	This work

	obtained by PCR with primers ALA-4 and REPDEL-2	
pDEL3	Ap^r, pBluescript SK+ with 348-bp *Sac*I-*Eco*RI fragment of *acmA* obtained by PCR with primers ALA-4 and REPDEL-3	This work
pGKAL1	Em^r, Cm^r, pGK13 containing *acmA* under control of its own promoter on a 1,942-bp *Ssp*I-*Bam*HI insert	(5)
pGKAL3	Em^r, Cm^r, pGKAL1 derivative expressing A2	This work
pGKAL4	Em^r, Cm^r, pGKAL1 derivative expressing A1	This work
pGKAL5	Em^r, Cm^r, pGKAL1 derivative expressing A0	This work
pGKAL6	Em^r, Cm^r, pGKAL1 derivative expressing A1.5	This work
pGKAL7	Em^r, Cm^r, pGKAL1 derivative expressing A4	This work
pETAcmA	Ap^r, pET32A expressing active site domain of AcmA from residues 58 to 218 fused to thioredoxin	This work
pGBL1	Em^r, pWV01 derivative expressing *E. coli* TEM-β-lactamase fused to export element BL1 of *L. lactis*	(21)
pGAL9	Em^r, pWV01 derivative expressing *B. licheniformis* α-amylase fused to export element AL9 of *L. lactis*	(21)
pUKAL1	Km^r, pUK21 with ±1,050-bp *Cla*I-*Hin*dIII fragment of pGAL9	This work
pUKAL2	Km^r, pUKAL1 in which the ±650-bp *Sal*I-*Eco*RV fragment is replaced by the 440-bp *Sal*I-*Eco*RV fragment of the PCR fragment obtained with primers ALFA-A and -B	This work
pUKALR	Km^r, pUKAL2 with 1,104-bp *Pvu*II-*Xba*I fragment of pAL01 in *Eco*RV and *Xba*I sites	This work

pUKblac	Km^r, pUK21 with 311-bp *PstI-NdeI* PCR fragment obtained with primers BETA-1 and -2	This work
pUKblacR	Km^r, pUKblac carrying 1,104-bp *PvuII-XbaI* fragment of pAL01 in *NdeI* and *XbaI* sites	This work
pGBLR	Em^r, pGBL1 expressing the β-lactamase/AcmA fusion protein	This work
pGALR	Em^r, pGAL9 expressing the α-amylase/AcmA fusion protein	This work

Table 2. Oligonucleotides used in this study[a)]

Name	Nucleotide sequence (5' → 3')	R/E site
REPDEL-1	CGCGAATTCAGA*TTA*TGAAACAATAAG	*Eco*RI
REPDEL-2	CGCGAATTC*TTA*TGTCAGTACAAGTTTTTG	*Eco*RI
REPDEL-3	CGCGAATTCC*TTA*TGAAGAAGCTCCGTC	*Eco*RI
ALA-4	CTTCAACAGACAAGTCC	
REP-4A	AGCAATACTAGTTTTATA	*Spe*I
REP-4B	CGCGAATTCGCTAGCGTCGCTCAAATTCAAAGTGCG	*Nhe*I
ACMHIS	AGGAGATCTGCGACTAACTCATCAGAGG	*Bgl*II
BETA-1	GGATCATGTAACTCGCC	
BETA-2	GGAATTCCATATGCTTAATCAGTGAGG	*Nde*I
ALFA-A	GCATCCGTTGAAAGCGG	
ALFA-B	GAATTCGATATCTTTGAACATAAATTG	*Eco*RV
ALA-14	GATAAATGATTCCAAGC	
ALA-22	CTCAAATTCAAAGTGCG	

a) The indicated restriction enzyme (R/E) sites are underlined while stopcodons are shown in italic.

Table 3. Properties of *L. lactis* expressing AcmA derivatives.

Number a)	Strain (plasmid) b)	AcmA variant c)	%Reduction in OD_{600} d)	PcpX activity e)	Chain lenght f)	Hole g)	Sedimentation h)	AcmA activity i)		Cell binding j)
								sup	ce	
1	MG pGK13	A3	32.6	16.9	A	3.1	-	+	+	+
2	*Δ1* pGK13	-	15.2	0.3	C	0	+	-	-	-
3	*Δ1* pGKAL1	A3	36.7	19.8	A	5.0	-	+	+	+
4	*Δ1* pGKAL3	A2	29.3	13.3	A	4.6	-	+	+	+
5	*Δ1* pGKAL4	A1	18.8	0.4	B	3.9	+	+	+	+
6	*Δ1* pGKAL5	A0	15.6	0.3	C	0	+	+	-	-
7	*Δ1* pGKAL6	A1.5	18.6	1.6	B	2.2	±	+	+	+
8	*Δ1* pGKAL7	A4	21.1	4.9	A	4.0	-	+	+	+

[a]) The number corresponds to the AcmA derivative produced, as schematized in Fig. 1.

[b]) MG: *L. lactis* MG1363, *Δ1*: *L. lactis* MG1363*acmAΔ1*.

[c]) -: no AcmA produced; Ax: AcmA with x repeats.

[d]) The OD_{600} reduction was calculated using the following formula: $[(OD_{max.}-OD_{60\ hours})/OD_{max.}]* 100\%$.

[e]) Activity is in arbitrary units measured as the increase of absorption at 405 nm in time.

[f]) End exponential phase ½M17 cultures were subjected to light microscopic analysis.

A: mainly single cells and some chains up to 5 cells

B: some single cells but mainly chains longer than 5 cells

C: no single cells, only very long chains

[g]) The sizes of the halo's were measured in millimeters from the border of the colony after 45 h of incubation at 30°C.

[h]) Analyzed by visual inspection of standing ½M17 cultures after overnight growth in test tubes.

[i]) Judged from zymograms of samples from end-exponential phase ½M17 cultures; sup: supernatant fraction, ce: cell-extract.

[j]) Binding of AcmA derivatives in supernatants of end-exponential phase ½M17 cultures to end-exponential phase cells of *L. lactis* MG1363*acmAΔ1* after 20 min of incubation at 30°C (see text for details).

FIG. 1. Analysis of AcmA activity in supernatant fractions of end-exponential-phase cultures of MG1363 containing pGK13 (1) and MG1363*acmAΔ1* containing either pGK13, not encoding AcmA (2), pGKAL1, encoding enzyme A3 (3), pGKAL3, encoding enzyme A2 (4), pGKAL4, encoding enzyme A1 (5), pGKAL5, encoding enzyme A0 (6), pGKAL6, encoding enzyme A1.5 (7), or pGKAL7, encoding enzyme A4 (8) in a renaturing SDS-(12.5%)PAA gel containing 0.15% *M. lysodeikticus* autoclaved cells. Molecular masses (in kilodaltons, kDa) of standard proteins (lane M) are shown in the left margin. Below the gel the lower part of lanes 5, 6 and 7 of the same gel is shown after one week of renaturation. The right half of the figure gives a schematic representation of the various AcmA derivatives. SS (black), signal sequence; Rx (dark grey), repeats; light grey, Thr, Ser and Asn-rich intervening sequences (6); arrows, artificially duplicated region in the AcmA derivative containing four repeats. The active site domain is shown in white. MW, expected molecular sizes in kDa of the secreted forms of the AcmA derivatives. The numbers of the AcmA derivatives correspond with the lane numbers of the gel. Numbered arrowheads indicate the putative location of proteolytic cleavage sites.

FIG. 2. Purification of the AcmA active site domain (nA). (**A**) SDS-(12.5%) PAGE of cell extract of 10 µl of *E. coli* BL21(DE3) (pETAcmA) (lane 3) induced for 4 h with IPTG. Lane 2, 10 µl of purified fusion protein isolated from 25 µl of induced *E. coli* culture and lane 1, 10 µl of the enterokinase cleft protein. (**B**) Renaturing SDS-(12.5%) PAGE with 0.15% *M. lysodeikticus* autoclaved cells using the same amount of the samples 1 and 2 shown in part A. Molecular masses (in kilodaltons) of standard proteins are shown on the left of the gel. Before loading the samples were mixed with an equal volume of 2x sample buffer (15).

FIG. 3. Schematic representation of plasmids pGBLR and pGALR carrying, respectively, C-terminal fusions of the repeats of AcmA to β-lactamase and α-amylase. *α-amy*, α-amylase gene of *B. licheniformis*; *β-lac*, β-lactamase gene of *E. coli*; *acmA*, 3-prime end of the *N*-acetylmuramidase gene of *L. lactis* MG1363 encoding the three repeats; *EmR* and *CmR*, erythromycin and chloramphenicol resistance genes; AL9 and BL1, protein secretion signals from *L. lactis* MG1363 (21); *repA* and ORI, gene for the replication protein and origin of replication of the lactococcal plasmid pWV01, respectively; Pspo2, *B. subtilis* phage Spo2 promoter. Black boxes indicate the PCR fragments used for the introduction of the restriction enzyme sites *Eco*RV and *Nde*I at the position of the stopcodons of the *α-amylase* and *β-lactamase* genes, respectively. The open box indicates the part which has been subcloned into pUK21 for construction work. The grey boxes show the fragment of pAL01 used to fuse the 3'-end of *acmA* to the α-amylase and β-lactamase genes. Only relevant restriction enzyme sites are shown.

FIG. 4. β-lactamase activity in *L. lactis*. Activity of wild-type β-lactamase and its AcmA fusion derivative (βcA) produced by cells of *L. lactis* MG1363 and MG1363*acmAΔ1* containing pGK13, pGBL1 or pGBLR. The ½M17 agar plate was stained with iodine after overnight growth of the colonies according the protocol of Smith *et al.* (29).

FIG. 5. α-amylase activity in the supernatant of *L. lactis*. Activity of wild-type α-amylase (α) and the αcA fusion protein in an SDS-(12.5%)PAA gel containing 1% starch. The proteins were renatured by washing the gel with Triton X-100 and subsequently stained with iodine (33). The equivalent of 40 μl of supernatant of ½M17 cultures of *L. lactis* MG1363 (M)

and MG1363*acmAΔ1* (Δ) containing pGAL9 or pGALR were loaded onto the gel. Molecular masses (in kDa) of standard proteins are shown in the left margin.

FIG. 6. Localization of β-lactamase in *L. lactis*. Western blot analysis of fractions of MG1363*acmAΔ1* expressing β-lactamase (from pGBL1) or βcA fusion protein (encoded by pGBLR) using polyclonal antibodies directed against β-lactamase. Amount of samples loaded is equal to 200 µl of culture. Fractions: S, supernatant; CW, cell wall; CY, cytoplasm; MB, membrane-associated; and M, membrane.

FIG. 7. Analysis of the binding of AcmA derivatives nA and A1 by a renaturing SDS-(17.5%) PAGE with 0.15% *M. lysodeikticus* autoclaved cells. Cell (C) and supernatant (S) fractions of MG1363*acmAΔ1* cells incubated with nA and A1 from the culture supernatant of MG1363*acmAΔ1* containing pGKAL4. 60 µl of the samples were loaded. Molecular masses (in kDa) of standard proteins are shown in the left margin.

FIG. 8. Binding of the βcA fusion protein to *L. lactis*. The figure shows a Western blot using polyclonal antibodies against β-lactamase. Cell extracts (lanes 1, 3, 5) and supernatants (lanes 2, 4, 6) of mid-exponential phase MG1363*acmAΔ1* (pGK13) cells incubated for 5 minutes with supernatants of MG1363*acmAΔ1* containing pGK13 (lanes 1, 2), pGBLR (lanes 3, 4) or pGBL1 (lanes 5, 6), respectively. The positions of wild-type β-lactamase (β) and the βcA fusion protein are indicated on the right. Molecular masses (in kDa) of standard proteins are shown in the left margin. Twenty µl of samples were loaded onto an 12.5% PAA gel.

FIG 9. Schematic representation of the AcmA protein. SS (black), signal sequence; R (dark grey), repeats; shaded regions, intervening sequences. The active site domain is shown in white.

FIG. 10. Amino acid sequence alignment of the repeats of AcmA in *L. lactis* plus consensus sequence.

Fig 11. Amino acid sequence alignment of repeats in various species

[a] Proteins listed were obtained by a homology search in the SWISSPROT, PIR, and Genbank databases with the repeats of AcmA using the BLAST program (1).

[b] * ; genes encoding cell wall hydrolases.
#' ; proteins containing repeats that are longer than average

[c] The number of aa residues between the repeats are given between brackets.

[d] Number of aa of the primary translation product.

[e] Genbank accession number.

References

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman**. 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403-410.

2. **Baankreis, R.** 1992. The role of lactococcal peptidases in cheese ripening. PhD Thesis. University of Amsterdam, the Netherlands.

3. **Béliveau, C., C. Potvin, J. Trudel, A. Asselin, and G. Bellemare**. 1991. Cloning, sequencing, and expression in *Escherichia coli* of a *Streptococcus faecalis* autolysin. J. Bacteriol. **173**:5619-5623.

4. **Birkeland, N.-K.** 1994. Cloning, molecular characterization, and expression of the genes encoding the lytic functions of lactococcal bacteriophage ØLC3: a dual lysis system of modular design. Can. J. Microbiol. **40**:658-665.

5. **Buist, G., H. Karsens, A. Nauta, D. Van Sinderen, G. Venema, and J. Kok**. 1997. Autolysis of *Lactococcus lactis* caused by induced overproduction of its major autolysin AcmA. Appl. Environ. Microbiol. **63**: **2722-2728**

6. **Buist, G., J. Kok, K.J. Leenhouts, M. Dabrowska, G. Venema, and A. J. Haandrikman**. 1995. Molecular cloning and nucleotide sequence of the gene encoding the major peptidoglycan hydrolase of *Lactococcus lactis*, a muramidase needed for cell separation. J. Bacteriol. **177**:1554-1563.

7. **García, J. L., E. Díaz, A. Romero, and P. García**. 1994. Carboxy-terminal deletion analysis of the major pneumococcal autolysin. J. Bacteriol. **176**:4066-4072.

8. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic Streptococci after protoplast-induced curing. J. Bacteriol. **154**:1-9.

9. **Hourdou, M.-L., M. Guinand, M.-J. Vacheron, G. Michel, L. Denoroy, C. Duez, S. Englebert, B. Joris, G. Weber, and J.-M.**

Ghuysen. 1993. Characterization of the sporulation-related γ-D-glutamyl-(L)*meso*-diaminopimelic-acid-hydrolysing peptidase I of *Bacillus sphaericus* NCTC 9602 as a member of the metallo(zinc) carboxypeptidase A family (Modular design of the protein). Biochem. J. **292**:563-570.

10. **Joris, B., S. Englebert, C. P. Chu, R. Kariyama, L. Daneo-Moore, G. D. Shockman, and J. M. Ghuysen**. 1992. Modular design of the *Enterococcus hirae* muramidase-2 and *Streptococcus faecalis* autolysin. FEMS Microbiol. Lett. **91**:257-264.

11. **Kariyama, R. and G. D. Shockman**. 1992. Extracellular and cellular distribution of muramidase-2 and muramidase-1 of *Enterococcus hirae* ATCC 9790. J. Bacteriol. **174**:3236-3241.

12. **Knowles, J., P. Lehtovaara, and T. Teeri**. 1987. Cellulase families and their genes. Tibtech. **5**:255-261.

13. **Kuroda, A. and J. Sekiguchi**. 1990. Cloning, sequencing and genetic mapping of a *Bacillus subtilis* cell wall hydrolase gene. J. Gen. Microbiol. **136**:2209-2216.

14. **Kuroda, A., Y. Sugimoto, T. Funahashi, and J. Sekiguchi**. 1992. Genetic structure, isolation and characterization of a *Bacillus licheniformis* cell wall hydrolase. Mol. Gen. Genet. **234**:129-137.

15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.

16. **Leenhouts, K. and G. Venema**. 1992. Molecular cloning and expression in Lactococcus. Med. Fac. Landbouww. Univ. Gent. **57**:2031-2043.

17. **Longchamp, P. F., C. Mauel, and D. Karamata**. 1994. Lytic enzymes associated with defective prophages of *Bacillus subtilis*: sequencing and characterization of the region comprising the *N*-acetylmuramoyl-L-alanine amidase gene of prophage PBSX. Microbiol. **140**:1855-1867.

18. **López, R., E. García, P. García, and J. L. García**. 1995. Architecture and domain interchange of the pneumococcal cell wall lytic enzymes. Dev. Biol. Stand. **85**:273-281.

19. **Oda, Y., R. Nakayama, A. Kuroda, and J. Sekiguchi**. 1993. Molecular cloning, sequence analysis, and characterization of a new cell wall hydrolase, CwlL, of *Bacillus licheniformis*. Mol. Gen. Genet. **241**:380-388.

20. **Oshida, T., M. Sugai, H. Komatsuzawa, Y. M. Hong, H. Suginaka, and A. Tomasz**. 1995. A *Staphylococcus aureus* autolysin that has an *N*-acetylmuramoyl-L-alanine amidase domain and an endo-b-*N*-acetylglucosaminidase domain: Cloning, sequence analysis, and characterization. Proc. Natl. Acad. Sci. USA **92**:285-289.

21. **Pérez Martínez, G., J. Kok, G. Venema, J. M. van Dijl, H. Smith, and S. Bron**. 1992. Protein export elements from *Lactococcus lactis*. Mol. Gen. Genet. **234**:401-411.

22. **Potvin, C., D. Leclerc, G. Tremblay, A. Asselin, and G. Bellemare**. 1988. Cloning, sequencing and expression of a *Bacillus* bacteriolytic enzyme in *Escherichia coli*. Mol. Gen. Genet. **214**:241-248.

23. **Rashid, M. H., M. Mori, and J. Sekiguchi**. 1995. Glucosaminidase of *Bacillus subtilis*: Cloning, regulation, primary structure and biochemical characterization. Microbiol. **141**:2391-2404.

24. **Ruhland, G. J., M. Hellwig, G. Wanner, and F. Feidler.** 1993. Cell-surface location of *Listeria*-specific protein p60: detection of *Listeria* cells by indirect immunofluorescence. J. Gen. Microbiol. **139**:609-616.

25. **Sambrook, J., E. F. Fritsch, and T. Maniatis**. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

26. **Sanger, F., S. Nicklen, and A. R. Coulson**. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463-5467.

27. **Sanz, J. M., P. García, and J. L. García**. 1996. Construction of a multifunctional pneumococcal murein hydrolase by module assembly. Eur. J. Biochem. **235**:601-605.

28. **Sheehan, M. M., J. L. García, R. López, and P. García**. 1996. Analysis of the catalytic domain of the lysin of the lactococcal bacteriophage Tuc2009 by chimeric gene assembling. FEMS Microbiol. Lett. **140**:23-28.

29. **Smith, H., S. Bron, J. van Ee, and G. Venema**. 1987. Construction and use of signal sequence selection vectors in *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. **169**:3321-3328.

30. **Studier, F. W. and B. A. Moffatt**. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189**:113-130.

31. **Towbin, H., T. Staechelin, and J. Gordon**. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350-4354.

32. **van De Guchte, M., J. Kodde, J. M. B. M. van der Vossen, J. Kok, and G. Venema**. 1990. Heterologous gene expression in *Lactococcus lactis* ssp. *lactis*: Synthesis, secretion, and processing of the *Bacillus subtilis* neutral protease. Appl. Environ. Microbiol. **56**:2606-2611.

33. **van Dijl, J. M., A. de Jong, J. Vehmaanpera, G. Venema, and S. Bron**. 1992. Signal peptidase I of *Bacillus subtilis*: patterns of conserved amino acids in prokaryotic and eukaryotic type I signal peptidases. EMBO J. **11**:2819-2828.

34. **Vasala, A., M. Valkkila, J. Caldentey, and T. Alatossava**. 1995. Genetic and biochemical characterization of the *Lactobacillus delbrueckii* subsp. *lactis* bacteriophage LL-H lysin. Appl. Environ. Microbiol. **61**:4004-4011.

35. **Vieira, J. and J. Messing**. 1991. New pUC-derived cloning vectors with different selectable markers and DNA replication origins. Gene **100**:189-194.

36. **Wilson, R., R. Ainscough, K. Anderson, C. Baynes, M. Berks, J. Bonfield, J. Burton, M. Connell, T. Copsey, J. Cooper, and et al**. 1994. 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans*. Nature **368**:32-38.

37. **Zabarovsky, E. R. and G. Winberg**. 1990. High efficiency electroporation of ligated DNA into bacteria. Nucl. Acid Res. **18**:5912.

38. **Stahl and Uhlen**, TIBTECH May 1997, 15, 185-192

39. WO 94/18830

40. WO 97/08553

41. **Medaglini et al.**, PNAS 1995, 2; 6868-6872

42. **Robinson et al.**, Nature Biotechnology 1997, 15; 653-657

Claims

1. A proteinaceous substance comprising at least one stretch of amino acids derived from a first micro-organism which substance is capable of attaching to a cell-wall of a second micro-organism, said stretch of amino acids having a sequence corresponding to a consensus sequence listed in figure 10.

2. A substance according to claim 1 wherein said stretch of amino acids has a sequence corresponding to a sequence selected from those listed in figure 11, or a homologous sequence derived from another species.

3. A substance according to claim 1 or 2 wherein said second micro-organism is a non-recombinant micro-organism.

4. A substance according to claim 3 wherein said second micro-organism is selected from any of the group of Gram-positive bacteria and Gram-negative bacteria.

5. A substance according to any one of claims 1 to 4 additionally comprising a reactive group.

6. A substance according to claim 5 wherein said reactive group is selected from the group of antibiotics, hormones, aromatic substances and reporter molecules.

7. A substance according to claim 5 wherein said reactive group is selected from the group of antigenic determinants, enzymes, (single-chain) antibodies or fragments thereof, polyhistidyl tags, fluorescing proteins, binding proteins or peptides.

8. A nucleic acid molecule encoding a proteinaceous substance according to claim 7.

9. A vector comprising a nucleic acid molecule according to claim 8.

10. A micro-organism or expression system comprising a nucleic acid molecule according to claim 8 or a vector according to claim 9 or expressing a substance according to any one of claims 1 to 5 or 7.

11. A method for attaching a substance to the cell wall of a micro-organism comprising the use of an attaching peptide

which comprises a stretch of amino acids having a sequence corresponding to the consensus amino acid sequence provided in figure 10.

12. A method for attaching a substance to the cell wall of a micro-organism comprising the use of an attaching peptide derived from any one of the proteins listed in figure 11.

13. A method according to claim 11 or 12 comprising the use of an attaching peptide which is derived from the major peptidoglycan hydrolase of *Lactococcus lactis*.

14. Use of a method according to any one of claims 11 to 13 to attach a substance to the cell-wall of a micro-organism.

15. A micro-organism to which a substance has been attached using a method according to any one of claims 11 to 13.

16. A pharmaceutical composition comprising a substance according to any of claims 1 to 7 or a micro-organism according to claim 10 or 15.

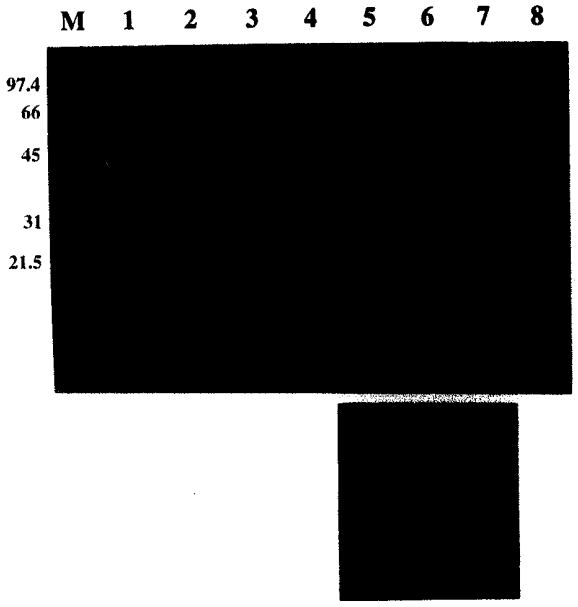
17. A vaccine comprising a substance according to any of claims 1 to 7 or a micro-organism according to claim 10 or 15.

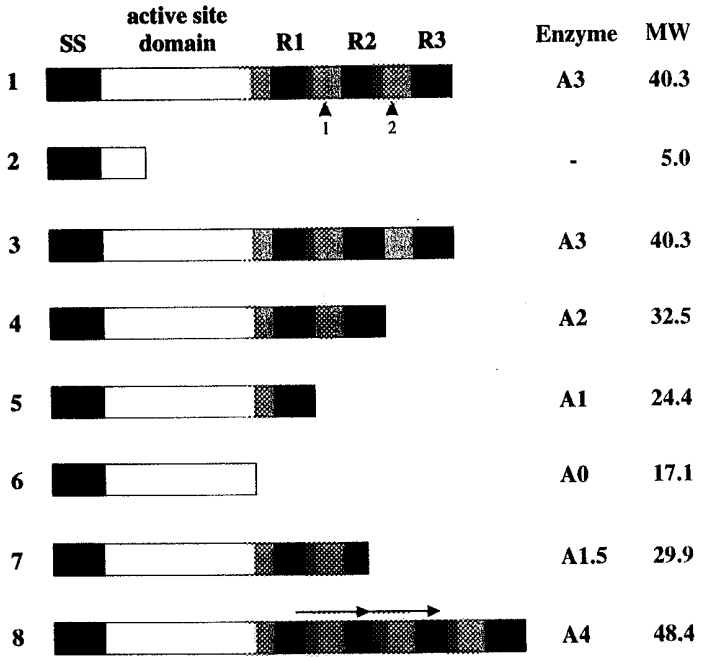
18. A foodstuff comprising a substance according to any of claims 1 to 7 or a micro-organism according to claim 10 or 15.

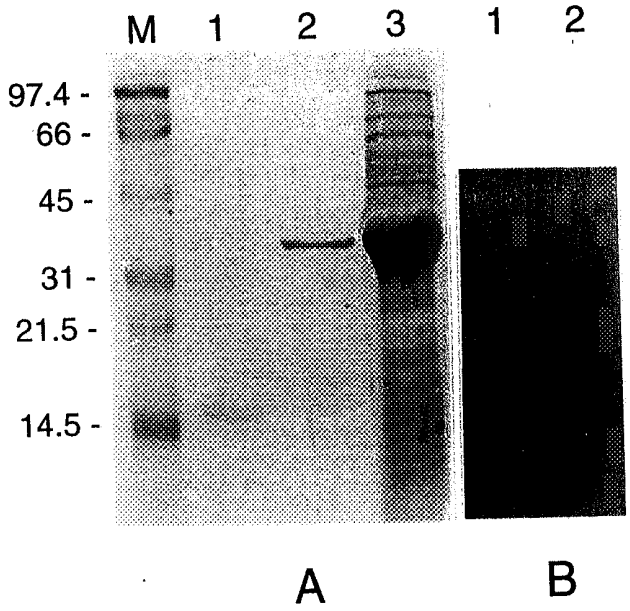
19. A process for the preparation of a foodstuff comprising the use of a substance according to any of claims 1 to 7 or a micro-organism according to claim 10 or 15.

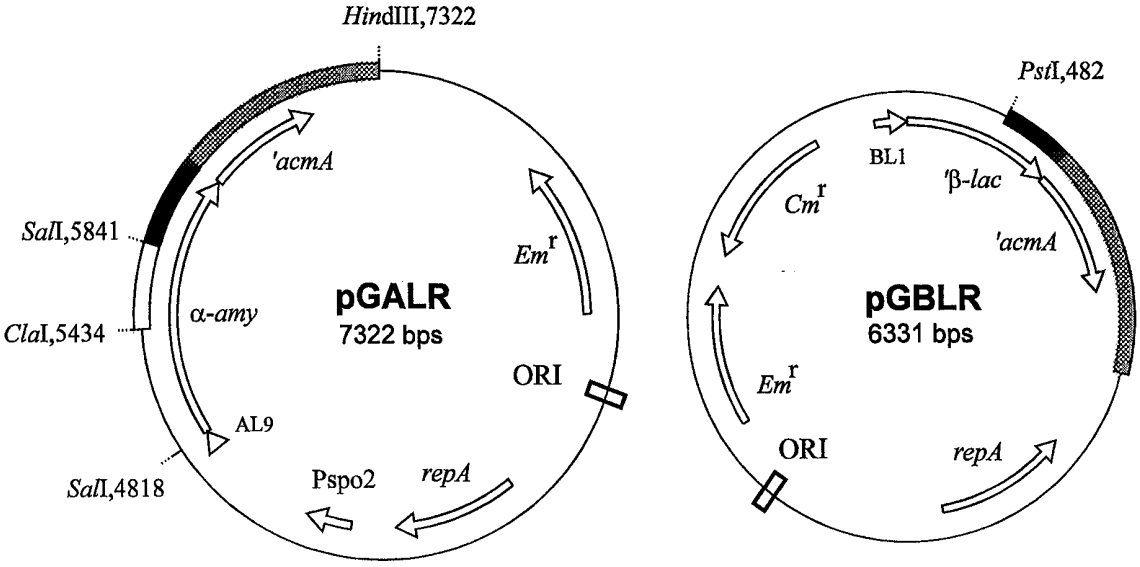
20. Use of a substance according to any of claims 1 to 7 or a micro-organism according to claim 10 or 15 in a diagnostic test.

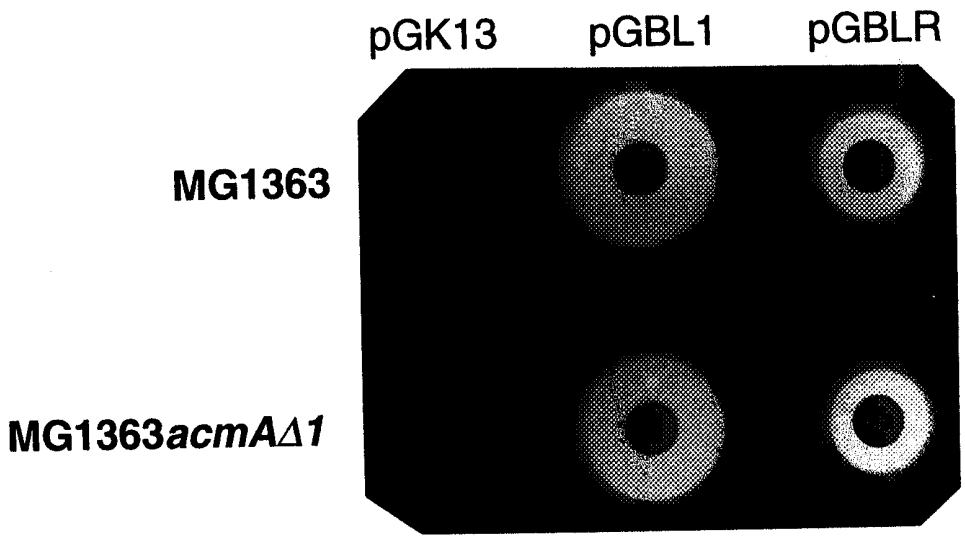
21. Use of a substance according to any of claims 1 to 7 or a micro-organism according to claim 10 or 15 in a bioadsorption process.

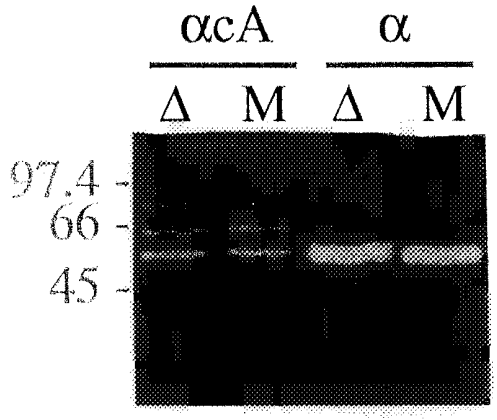

M 1 2 3 4 5 6 7 8
97.4
66
45
31
21.5

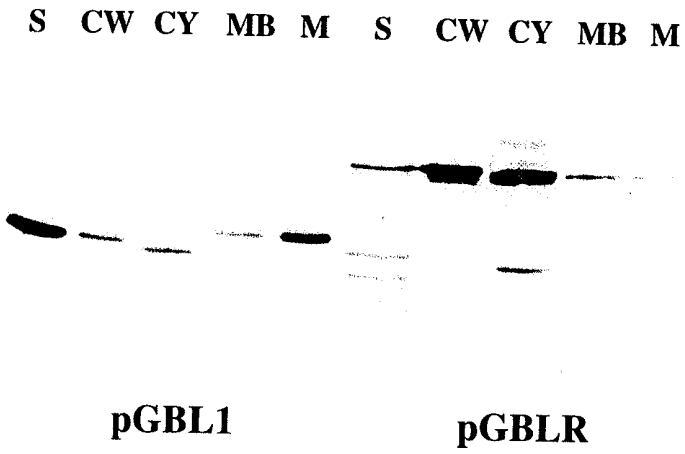

SS
active site domain
R1
R2
R3
1
2
Enzyme
MW
1 A3 40.3
2 - 5.0
3 A3 40.3
4 A2 32.5
5 A1 24.4
6 A0 17.1
7 A1.5 29.9
8 A4 48.4

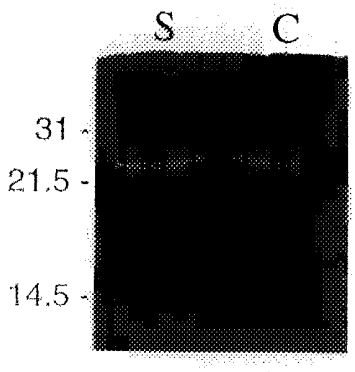

M
1
2
3
1
2
97.4 -
66 -
45 -
31 -
21.5 -
14.5 -
A
B

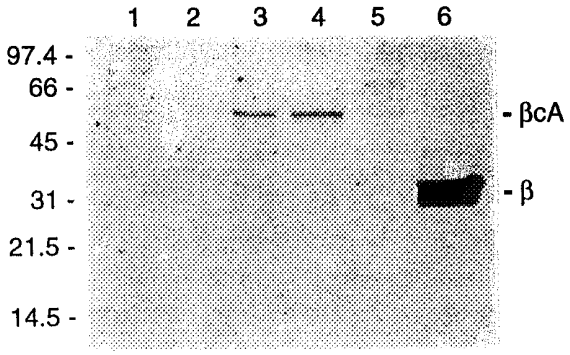

HindIII,7322
'acmA
SalI,5841
ClaI,5434
α-amy
pGALR
7322 bps
Em r
ORI
AL9
SalI,4818
Pspo2
repA
PstI,482
BL1
'β-lac
Cm r
'acmA
pGBLR
6331 bps
Em r
ORI
repA

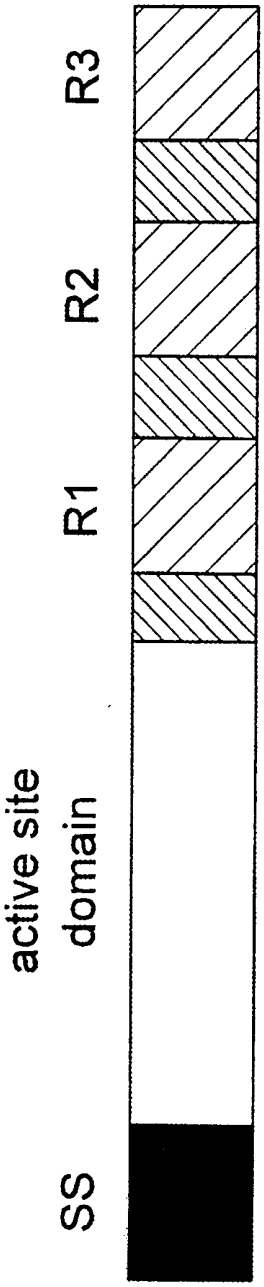

pGK13
pGBL1
pGBLR
MG1363
MG1363acmAΔ1


αcA
α
Δ M Δ M
97.4
66
45


S CW CY MB M S CW CY MB M
pGBL1
pGBLR


S
C
31
21.5
14.5


1
2
3
4
5
6
97.4 -
66 -
45 -
31 -
21.5 -
14.5 -
- βcA
- β


R3
R2
R1
active site
domain
SS

```
R1 TTYTVKSGDTLWGISQRYGISVAQIQSANNLKSTIIYIGQKLVLT  243-287
R2 TTVKVKSGDTLWALSVKYKTSIAQLKSWNHLSSDTIYIGQNLIVS  319-363
R3 SIHKVVKGDTLWGLSQKSGSPIASIKAWNHLSSDTILIGQYLRIK  393-437
     .. .* .****** .* .   ..* .... *.*.*..* *** * ..

Consensus:
```

Lactococcus lactis	* *acm4*	YTVKSGDTLWGISQRYGISVAQISAN NLKST IIYIGQKLVLT	245-287(33)	437	U17696	muramidase
		VKVKSGDTLWALSVRKYKTSIAQLKSWN HLSSD TIYIGQNLIVS	321-363(31)			
		HKVVKGDTLWGLSQKSGSPIASIKAWN HLSSD TILIGQYLRIK	395-437			
Enterococcus faecalis	* autolysin	YTVKSGDTLNKIAAQYGVSVANLRSWN GISGD LIFVGQKLIVK	363-405(25)	671	P37710	muramidase
		YTVKSGDTLNKIAAQYGVTVANLRSWN GISGD LIFVGQKLIVK	431-473(25)			
		YTIKSGDTLNKIAAQYGVSVANLRSWN GISGD LIFAGQKIIVK	499-541(25)			
		YTIKSGDTLNKISAQFGVSVANLRSWN GIKGD LIFAGQTIIVK	567-609(19)			
		HTVKSGDSLWGLSMQYGISIQKIKQLN GLSGD TIYIGQTLKVG	629-671			
Enterococcus hirae	* *mur2*	YTVKSGDSVWGISHSFGITMAQLIEWN NIKNN FIYPGQKLTIK	257-299(38)	666	P39046	muramidase
		YTVKSGDSVWKIANDHGISMNQLIEWN NIKNN FVYPGQQLVVS	338-380(33)			
		YTVKAGESVWSVSNKFGISMNQLIQWN NIKNN FIYPGQKLIVK	414-456(32)			
		YTVKAGESVWGVANKNGISMNQLIEWN NIKNN FIYPGQKLIVK	489-531(33)			
		YTVKAGESVWGVANKHHITMDQLIEWN NIKNN FIYPGQEVIVK	565-607(15)			
		YTVKAGESVWGVADSHGITMNQLIEWN NIKNN FIYPGQQLIVK	623-665			
Lactococcus Ø*Tuc2009*	* *lys*	YVVKQGDTLSGIASNWGTNWQELARQN SLSNFNMIYAGQVISFT	332-375(10)	428	L31364	glycosidase (muramidase)
		YTVQSGDNLSSIAILLGTTVQSLVSWN GISNFNLIYAGQTLNY	386-428			
Lactococcus Ø*-LC3*	* *lysB*	YIVKQGDTLSGIASNLGTNWQELARQN SLSNFNMIYSGQVISLT	333-376(10)	429	U04309	muramidase
		YTVQSGDNLSSIARRLGTTVQSLVSWN GISNFNLIYAGQTLNV	387-429			
Bacillus ØPBSX	* *xylA*	YVVKQGDTLTSIARAFGVTVAQLQEWN NIEDFNLIRVGQVLIVS	161-204	297	P39800	*N*-acetylmuramoyl-L-alanine amidase
Bacillus Ø PZA (=Ø-29)	* *orf15*	YKVKSGDNLTKIAKEHNTTVATLKLNFPSIKDFNMIRVGQTINVT	163-207(6)	258	P11187	lysozyme
		HVVKGDTLSEIAKKIKTSTKTLLELNFTIKNFNKIYVGQRINVG	214-258		P07540	
Bacillus Ø B103	* *orf15*	YKIKRGETLTGIAKKNKTTVSQLMKLNFNNIKNANYIYAGQTIRLK	219-263	263	X99260	lysozyme
		YTVVSGDSWWKIAQRNGLSMYTLASQN GKSIYSTIYPGNKLIIK	399-442	442	1926389	lysin
Bacillus Øgle	* *lys*	ILIRPGDSLWYFSDLFKIPLQLLDDSNRNINPQ LLQVGQRIQIP	3- 46(6)	396	X69507	carboxypeptidase I
Bacillus sphaericus	* Pep I	YTITQGDSLWQIAQNKNLPLNAILLVNPETQPS RLHIGQTIQVP	53- 96			
Haemophilus influenzae	* *amiB*	HIVKKGESLGSLSNKYHVKVSDIIKLN QLKRK TLWLNESIKIP	294-336	432	P44493	*N*-acetylmuramoyl-L-alanine amidase
		HKVTKNQTLYAISREYNIPVNILLSLNPHLKNG KVITQGKIKLR	387-430			
Listeria monocytogenes	* P60	VVVEAGDTLWGIAQSKGTTVDAIKKAN NLTTD KIVPGQKLQVN	30- 72(130)	478	M80351	adherence and invasion protein P60
		HAVKSGDTIWALSVKYGVSVQDIMSWN NLSSS SIVVGQKLAIK	203-245			
Listeria innocua	* P60	VVVEAGDTLWGIAQSKGTTVDAIKKAN NLTTD KIVPGQKLQVN	30- 72(130)	481	Q01836	adherence and invasion protein P60
		HNVKSGDTIWALSVKYGVSVQDIMSWN NLSSS SIYVGQKPAIK	201-243			
Listeria ivanovii	* P60	VVVEAGDTLWGIAQDKGTTVDALKKAN NLTSD KIVPGQKLQIT	30- 72(125)	524	Q01837	adherence and invasion protein P60
		YTVKSGDTIWALSSKYGTSVQNIMSWN NLSSS SIVVGQVLAVK	198-240(73)			
		YTVKSGDTLSKIATTFGTTVSKIKALN GLNSD NLQVGQVLKVK	314-356			
Listeria seeligeri	* P60	VVVEAGDTLWGIAQDNGTTVDALKKAN KLTTD KIVPGQKLQVT	30- 72(127)	523	Q01838	adherence and invasion protein P60
		HTVKSGDTIWALSVKYGASVQDLMSWN NLSSS SIVVGQNIAVK	200-242(75)			
		YTVKSGDTLGKIASTFGTTVSKIKALN GLTSD NLQVGDVLKVK	320-362			
Listeria welshimeri	* P60	VVVEAGDTLWGIAQSKGTTVDALKKAN NLTSD KIVPGQKLQVT	30- 72(127)	524	M80348	adherence and invasion protein P60
		HTVKSGDTIWALSVKYGASVQDLMSWN NLSSS SIVVGQKIAVK	198-240(75)			
		YTVKSGDSLSKIANTFGTSVSKIKALN NLTSD NLQVGTVLKVK	316-358			
Listeria grayi	* P60	VVVASGDTLWGIASKYGTTVDQLKQLN KLDSD RIVPGQKLTIK	30- 72(104)	511	Q01835	adherence and invasion protein P60
		YKVKSGDTTWALSVKYGVPVQKLIEWN NLSSS SIVVGQTIAVK	177-219(79)			
		YKVQNGDSLGKIASLFKVSVADLTNWN NLNATTIYAGQELSVK	299-342			
Escherichia coli	* *yebA*	YVVSTGDTLSSILNQYGIDMGDISQLAAADKELRNLKIGQQLSWT	77-121	419	P24204	homologous to endopeptidase of *Staphylococcus*
Haemophilus influenzae	* *yebA*	YTVTEGDTLKDVLVLSGLDDSSVQPLIALDPELAHLKAGQQFWWI	131-174	475	P44693	homologous to endopeptidase of *Staphylococcus*
Escherichia coli	*nlpD*	YTVKKGDTLFYIAWITGNDFRDLAQRN NIQAPYALNVGQQTLQVG	123-166	379	P33648	Lipoprotein
Haemophilus influenzae	*lppB*	YKVNKGDTMFLIAYLAGIDVKELAALNNLSEFNYNLSLGQVLKIS	147-190	405	P44833	Lipoprotein
Haemophilus somnus	*lppB*	YKVRKGDTMFLIAYISGMDIKELATLN NMSEPYHLSIGQVLKIA	120-164	279	L10653	Lipoprotein
Pseudomonas aeruginosa	*lppB*	YIVRRGDTLYSIAFRFGWDWKALAARN GIAPPYTIQVGQAIQFG	69-113	297	P45682	Lipoprotein
Synechocystis	*nlpD*	HQVKEGESLWQISQAFQVDAKAIALAN SISTDTELQAGQVLNIP	87-130	715	D90915	Lipoprotein
Sinorhizobium meliloti	*nlpD*	IMVRQGDTVTVLARRFGVPEKEILKAN GLKSASQVEPGQRLVIP	166-209	512	U81296	Lipoprotein
Escherichia coli	*dniR*	YTVRSGDTLSSIASRLGVSTKDLQQWN KLRGS KLKPGQSLTIG	113-155(16)	222	P23931	involved in hexaheme nitrite reductase expression
		YRVRKGDTLSSIAKRHGVNIKDVMRWN SDTA NLQPGDKLTLF	172-213			
Staphylococcus aureus	ProtA	HVVKPGDTVNDIAKANGTTADKIAADN KLADKNNIKPGQELVVD	431-474	524	A04512	protein A
Bacillus subtilis	*papQ*	IKVKKGDTLWDLSRKYDTTISKIKSEN HLRSD IIYVGQTLSIN	28- 70(17)	334	U38819	phophatase-associated protein, P60 homolog
		IKVKSGDSLWKISKKYGMTINELKKLN GLKSD LLRVGQVLKLK	88-130(20)			
		YKVKSGDSLSKIASKYGTTVSKLKSLN GLKSD VIVVNQVLKVK	151-193			
Bacillus subtilis	*spoVID*	CIVQQEDTIERLCERYEITSQQLIRWN SLALDDELKAGQILYIP	525-568	575	P37963	stage VI sporulation protein D
Escherichia coli		YTVKRGDTLYRISRTTGTSVKELARLN GISPTYTIEVGQKLKLG	50- 93	259	U28375	Hypothetical protein
Synechocystis sp.		HVVKAGETIDSIAAQYQLVPATLISVN NQLSSGQVTPGQTILIP	4- 47	245	D90907	Hypothetical protein
Bacillus subtilis	*yaaH*	MVKQGDTLSAIASQYRTTTNDITEIN EIPNPDSLVVGQTTVIP	1- 43(5)	427	p37531	Hypothetical protein
		YDVKRGDTLTSIARQFNTTAAELARVN RIQLNTVLQIGPRLYIP	49- 92			
Bacillus subtilis	*yhdD*	IKVKSGDSLWKLAQTYNTSVAALTSAN HLSTT VLSIGQTLTIP	29- 71	488	2226145	Hypothetical protein
		YTVKSGDSLWLIANEFKMTVQELKKLN GLSSD LIRAGQKLKVS	94-136(29)			
		YKVQLGDSLWKIANKYNMSIAELKVLN NLKSD TIVVNQVLKTK	176-218(23)			
		YTVKSGDSLWKIANNYNLTVQQIRMIN NLKSD VLVVGQVLKLT	242-284(24)			
		YTVKSGDSLWVIAQKFNVTAQQIREKN NLKTD VLGVGQKLVIS	309-353			

Caenorhabditis elegans		TEIKSGDSCWNIASNAKISVERLQQLN KGNKCDKLPLGDKLCLA	23- 66(11)	1614	U64836	PUTATIVE ENDOCHITINASE
		LKLKAEDTCFKIWSSQKLSERQFLGNN EGNDCDKLKVGKEVCVA	78-121(21)			
		HKIQKGDTCFKIWTTNKISEKQFRNLN KGLDCDKLEIGKEVCIS	143-186(21)			
		LKIKEGDTCYNIWTSQKISEQEFMELN KGLDCDKLEIGKEVCVT	208-251(19)			
		YRFKKGDTCYKIWTSHKMSEKQFRALN RGIDCDRLVPGKELCVG	271-314(20)			
		ITVKPGDTCFSIWTSQKMTQQQFMDIN PELDCDKLEIGKEVCVT	335-378(23)			
		VKINPGDTCFNIWTSQRMTQQQFMDLN KRLDCDKLEVGKEVCVT	402-445(21)			
		VQINPGDTCFKIWSAQKLTEQQFMELN KGLDCDRLEVGKEVCIA	467-510(37)			
		TEVKEGDTCFKIWSAHKITEQQFMEMN RGLDCNRLEVGKEVCIV	548-591(44)			
		IKVKEGDTCFKIWSAQKMTEQQFMEMN RGLDCNKLMVGKEVCVS	636-679(66)			
		ATITPGNTCFNISVAYGINLT DLQ KTYDCKALEVGDTICVS	746-786(8)			
		IEVIKGDTCWFLENAFKTNQTEMERAN EGVKCDNLPIGRMMCVW	795-838			
Caenorhabditis elegans		HTIKSGDTCWKIASEASISVQELEGLN SKESCANLAVGLSEQEF	23- 66(51)	1484	U70858	PUTATIVE ENDOCHITINASE
		IHVKEGDTCYTIWTSQHLTEQFMDMN EELNCGHLEIGNEVCVD	118-161(25)			
		ATVTPGSSCYTISASYGLNLAELQTTY NCDALQVDDTICVS	187-226(9)			
		IELNGDTCGFLENAFQTNNTEMEIAN EGVKCDNLPIGRMMCVW	236-279			
Bacillus subtilis	#	HTVQKKETLYRISMKYYKSRTGSEKIRAYNHLNGNDVYTGQVLDIP	191-136	240	L47648	
Citrobacter freundii	# eae	YTLKTGESVAQLSKSQGISVPVIWSLNKHLYSSESEMMKASPGQQIILP	65-113	936	Q07591	
Escherichia coli	# eae	YTLKTGETVADLSKSQDINLSTIWSLNKHLYSSESEMMKAAPGQQIILP	65-113	934	P43261	
Bacillus subtilis	# yneA	IEVQQGDTLWSIADQVADTKKINKNDFIEWVADKNQLQTSDIQPGDELVIP	40- 90	105	Z73234	
Streptococcus pyogenes	#	YTVKYGDTLSTIAEAMGIDVHVLGDINNIANIDLIFPDTILTANYNQHGQATTLT	47-103	339	U09352	
Bacillus subtilis	# ydbp	YTVKKGDTLWDIAGRFYGNSTQWRKIWNANKTAMIKRSKRNIRQPGHWIFPGQKLKIP	177-234	235	G1225954	
Bacillus subtilis	#	YTVKKGDTLWDLAGKFYGDSTKWRKIWKVNKKAMIKRSKRNIRQPGHWIFPGQKLKIP	161-218	219	P45932	

INTERNATIONAL SEARCH REPORT

Internat .l Application No
PCT/NL 98/00655

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/31 C12N1/20 C07K14/315 C07K14/195 C07K14/37
C12N9/36 A61K38/02 A23L1/03 G01N33/68 B01J20/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 127, no. 3, 21 July 1997 Columbus, Ohio, US; abstract no. 31403, PIARD, J.-C. ET AL: "Cell wall anchoring of the Streptococcus pyogenes M6 protein in various lactic acid bacteria" XP002063307 see abstract & J. BACTERIOL. (1997), 179(9), 3068-3072 CODEN: JOBAAY;ISSN: 0021-9193, ---	1
Y	US 5 616 686 A (FISCHETTI VINCENT A ET AL) 1 April 1997 see claims --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 April 1999	13/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Delanghe, L

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internat al Application No
PCT/NL 98/00655

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 112, no. 7, 12 February 1990 Columbus, Ohio, US; abstract no. 51896, PANCHOLI, VIJAYKUMAR ET AL: "Identification of an endogenous membrane anchor-cleaving enzyme for group A streptococcal M protein. Its implication for the attachment of surface proteins in Gram-positive bacteria" XP002063308 see abstract & J. EXP. MED. (1989), 170(6), 2119-33 CODEN: JEMEAV;ISSN: 0022-1007, ---	1
Y	CHEMICAL ABSTRACTS, vol. 106, no. 13, 30 March 1987 Columbus, Ohio, US; abstract no. 98247, TAYLOR, M. J. ET AL: "Is there evidence for a common amino acid sequence of proteins with membrane attaching ability?" XP002063309 see abstract & J. THEOR. BIOL. (1986), 121(3), 293-306 CODEN: JTBIAP;ISSN: 0022-5193, ---	1
Y	CHEMICAL ABSTRACTS, vol. 123, no. 9, 28 August 1995 Columbus, Ohio, US; abstract no. 106450, BUIST, GIRBE ET AL: "Molecular cloning and nucleotide sequence of the gene encoding the major peptidoglycan hydrolase of Lactococcus lactis, a muramidase needed for cell separation" XP002063310 see abstract & J. BACTERIOL. (1995), 177(6), 1554-63 CODEN: JOBAAY;ISSN: 0021-9193, ---	1
Y	WO 95 31561 A (QUEST INT ;BUIST GIRBE (NL); VENEMA GERARD (NL); KOK JAN (NL); LED) 23 November 1995 see claims ---	1
Y	WO 95 09232 A (ALSTYNE DIANE VAN ;SHARMA LAWRENCE RAJENDRA (CA)) 6 April 1995 see claims -----	1

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna al Application No
PCT/NL 98/00655

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5616686 A	01-04-1997	US 5821088 A	13-10-1998
		US 5786205 A	28-07-1998
WO 9531561 A	23-11-1995	AU 2353995 A	05-12-1995
		EP 0759998 A	05-03-1997
		JP 10500012 T	06-01-1998
WO 9509232 A	06-04-1995	US 5510264 A	23-04-1996
		AU 7650494 A	18-04-1995
		EP 0677101 A	18-10-1995
		US 5556757 A	17-09-1996